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ISOLATED CHROMOSOMES

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The chromatin threads, which we described briefly several years ago (1), have since then been studied carefully both morphologically and chemically. We now consider these microscopic threads of chromatin to be isolated chromosomes (2). From certain animal cells such masses of isolated chromosomes can be prepared that, with respect to quantity of material available, no special methods are required for chemical investigation. It need hardly be said that interest in the chemical properties of this material is greatly enhanced by the knowledge that the material consists of chromosomes, for this provides the chemical studies with a background acquired by several generations of cytological investigation of chromosomes. In this paper the preparation of isolated chromosomes is described and evidence is presented that the bodies isolated are indeed chromosomes.

Preparation of Isolated Chromosomes.—Chromosomes were first isolated from fish erythrocytes and their isolation from this source is simpler than from any other. Erythrocytes of the salmon and carp have been used. Of these the salmon is to be preferred, being a much larger fish, but for most investigators the carp is far more readily available. In some cities large, live carp can be had in the fish stores. Blood is withdrawn from the caudal vein with a syringe, mixed with oxalate, and centrifuged for 15 minutes at 5000 R.P.M. A white sediment, consisting of leucocytes, is obtained. The red cells are mixed with three volumes of 0.14 M NaCl and centrifuged. This time the sediment consists of red cells. The red cells are well washed with saline. Finally, suspended in a large volume of saline, they are placed in a Waring mixer for 4 minutes. All the cells and nearly all the nuclei are broken by this treatment, so that on centrifuging a clear red supernatant and a nearly colorless precipitate are obtained. The precipitate, suspended in saline, is placed in the mixer for 1 minute and centrifuged again. Washing is continued in this manner until the supernatant is colorless and at this time the precipitate is also nearly colorless. It consists of chromosomes isolated from the erythrocyte nucleus (Figs. 5 and 6). The whole procedure is carried out in a cold room kept at about 1°C.

The procedure that has just been described is nothing else than the first part

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of the method we have used for the preparation of chromosin (a desoxyribose nucleoprotein complex derived from the cell nucleus) from liver, kidney, and other tissues (3); and chromosomes were first prepared from salmon blood, much to our own surprise, while we were in the process of preparing chromosin from the nuclei of salmon erythrocytes. After our publication of a brief description of chromatin threads the paper by Claude and Potter appeared giving an account of their work, done altogether independently of ours, on chromatin threads (4). Their method of isolating threads is different from ours, but they mention the use of the Waring mixer and they say that this machine destroys the threads. We do not know why Claude and Potter found the Waring high speed mixer unsatisfactory; in our experience with it we have never failed to obtain chromatin threads.

A more readily available material than fish blood for isolation of chromosomes is calf thymus, and this is certainly the material of choice if large masses of chromosomes are to be prepared. Preparation is not as simple as from fish erythrocytes because the cytoplasm is not as easily removed as is the cytoplasm of an erythrocyte, consisting as it does of almost nothing but hemoglobin. The thymus is minced with scissors, suspended in 0.14 M NaCl, and poured into a Waring mixer. For each 500 cc. of saline 125 gm. of minced thymus are used. To break the nuclei it is necessary for the material to remain in the mixer considerably longer than the 4 minutes which suffice to break most of the nuclei in a suspension of fish erythrocytes. When the mixer operates for even 4 minutes its contents warm up and, after running for 15 to 20 minutes the material in the mixer would be ruined by the heat produced. To keep the material cool it can be removed from the mixer every 4 minutes and cooled in an ice bath. A more satisfactory way of keeping the material cold is to construct a refrigerated high speed mixer. The one we have had made is of the same size and shape as the Waring mixer, but it is made of stainless steel instead of glass. Around the vessel is a jacket which contains crushed ice immersed in alcohol. To break practically all the nuclei in minced thymus a run of 20 minutes at high speed is required.

The chromosomes suspended in the debris of disintegrated thymus are separated from large particles by straining and from small, light particles by centrifugation. The suspension of disintegrated tissue, to which a drop of octyl alcohol has first been added to cut down the foam, is first centrifuged for 10 minutes at 3500 R.P.M. and the supernatant is discarded The precipitate is taken up in 750 cc. of saline and broken up in the Waring mixer, now run at low speed by connecting it with a rheostat. This suspenson is strained through a double layer of finely woven towel and then centrifuged at 3000 R.P.M. for 5 minutes. The precipitate is again resuspended in the mixer at low speed and then strained through a double layer of the finest muslin. It is centrifuged at 2500 R.P.M. for 10 minutes, resuspended, and again centrifuged. This process is repeated once more. Under the microscope nothing but chro-

mosomes and a few nuclei can be seen (Fig. 13). Continuing the washing
process does not increase the phosphorus content (in this case the measure of
nucleic acid content), and this is a sign that particles lighter than chromosomes
are no longer being removed. Suspended in saline in the cold, isolated chro-
mosomes can be kept for 3 or 4 days.

Isolated chromosomes have also been prepared from fowl erythrocytes.
When washing the erythrocytes, care is taken to remove all of the buffy coat
of leucocytes. The suspension in saline of washed erythrocytes is placed in
the refrigerated high speed mixer for 20 minutes. No straining is needed;
washing is entirely by centrifugation. In some preparations the precipitate
of chromosomes, instead of being almost colorless as when prepared from fish
erythrocytes or calf thymus, contains a brown pigment and also many intact
nuclei are present. Preparations from chicken erythrocytes have in general
not been as satisfactory as those from fish erythrocytes.

Chromosomes have also been isolated from mammalian liver (Figs. 3, 4, 9,
10) but for this material the procedure followed for isolation of thymus chro-
mosomes is unsatisfactory. Suspensions of liver chromosomes are obtained
easily enough; the difficulty comes in completely separating the chromosomes
from cell debris. For this purpose it has been necessary to modify the pro-
cedure, and this modification will be described in another paper.

Since nuclei, free of cytoplasmic debris, can be isolated from liver and many
other tissues by the use of citric acid, it might be thought that a simple way to
prepare chromosomes from these sources would be to break up the isolated
nuclei. Preparation of chromosomes from nuclei isolated by the citric acid
technique is, however, beset by two difficulties: while suspended in citric acid,
nuclei are not fragmented even after prolonged runs in the high speed mixer;
and if the citric acid is neutralized or washed away, the nuclei may, with diffi-
culty, be broken, but the chromosomes that are liberated are badly deformed.

When cells are subjected to a powerful shearing force, as they are in a high
speed mixer, the way in which they are fragmented is markedly influenced by
the pH of the medium. In a neutral medium, the cell membrane is broken
first, and as the shearing process is continued, more and more nuclei are frag-
mented. When the pH of the medium is lowered to 5, there still is no difficulty
in breaking the cell membrane, but the nucleus is fragmented with great diffi-
culty. At pH 3 the shearing forces we have applied do not break nuclei, al-
though cells are easily broken. It should be noted that even in the range be-
tween pH 6 and 7 (and it is in this pH range that our preparations of chromo-
somes have been made) some nuclei always remain in the final preparation of
chromosomes when a Waring mixer is used.

Disruption of the cell, it may be assumed, affects the composition of chromo-
somes isolated from it. Such effects are grouped under the general term of
"autolysis." Experiments, some of which will be described in another paper
(5), do in fact show that autolysis occurs in both isolated nuclei and chromo-

somes. These autolytic processes increase rapidly as the pH of the medium is raised. For this reason it is desirable to isolate chromosomes at a faintly acid reaction—but not acid enough to prevent nuclear fragmentation. It need hardly be said that in addition to autolytic changes which we can recognize and attempt to keep at a low level, isolation of chromosomes will also be attended by some morphological changes.

In most of our work the instrument used for fragmentation of cells has been a Waring mixer. Recently, at the suggestion of Dr. James Bonner, we have used a colloid mill (a Premier Mill, Type L-7). In this machine, as in the mixer, shearing forces are employed; indeed the colloid mill may be considered to be essentially a continuous-flow mixer. For isolation of chromosomes a colloid mill is far superior to a mixer, for the following reasons: the shearing force employed is greater, so that it is possible to break virtually all the nuclei in a cell suspension; action is more rapid, so that autolytic changes are reduced; rises in temperature are more readily prevented, and this is important in reducing autolysis.

Microscopic Study

For the investigation of these threads it is of considerable interest whether they are merely unspecific nucleoprotein fibers or isolated chromosomes. Microscopic analysis makes it certain that we are dealing with isolated chromosomes.

Chromosomes are characterized by the following features: (1) They show a definite organization along their axis such as primary and secondary constrictions, trabants, heterochromatic and euchromatic sections. (2) This pattern is specific for each chromosome at least within the same type of cells. (3) Chromosomes consist of a more or less tightly coiled helix, which can be uncoiled by special treatment. (4) Chromosomes consist of at least two chromonemata coiled together.

The threads isolated from nuclei show all these features and must therefore be regarded as chromosomes. They vary greatly in size and organization. Usually their doubleness can be clearly seen (Fig. 1). They show a specific pattern, with trabants, heterochromatic and euchromatic sections (Figs. 2, 3, 5, 10, 12). Like chromosomes they can be uncoiled by certain agents, KCN for example (Figs. 7, 8). These characteristics by themselves are strong evidence for the chromosome nature of these threads. Final proof, however, lies in the repeated occurrence of one and the same type of chromosome. Figs. 11 and 12 show two chromosomes of the same type isolated from calf thymus. Figs. 9 and 10 represent two chromosomes of the same type from calf liver.

The chromosome suspensions prepared by the method described are remarkably free of non-chromosomal material. A drop of such a suspension can be smeared on a slide and stained with Feulgen and light green. All non-

chromosomal material will then appear green. Except for nucleoli there is practically no extrachromosomal material present. The nucleoli are small in bulk compared with the chromosomes. They can be seen on such a smear either as separate particles or still attached to a chromosome.

In the tissues used for the preparation of these chromosomes only very few cells are in mitosis. The isolated chromosomes therefore are clearly liberated from resting nuclei. Cytologists have known for some time, mainly from indirect evidence, that chromosomes persist as individuals through interphase. But here we have very clear direct evidence to this effect, and the morphology of these chromosomes, probably only slightly altered, can be studied microscopically.

Claude and Potter (4) isolated chromatin threads from mouse leucemic cells and suggested that they represented chromosomes. However, their arguments do not favor their conclusion but rather indicate the opposite. They say (p. 349):

"Comparative study of the isolated chromatin threads with stained nuclei of the sort from which they were obtained is highly suggestive of a relationship to preformed nuclear structures. A close inspection of the nuclei represented in Fig. 1 shows, especially in the best preserved specimen, a number of delicate strands crossing the nuclear framework which, in their appearance, are not unlike the beaded filaments seen in Fig. 7. It is impossible to judge the length of the chromatin threads in the resting nucleus, but no loose ends are seen in the chromatin strands of nuclei of Fig. 2, which may imply the existence of a continuous filament or end-to-end arrangement of individual segments of chromatin. On the other hand a fair agreement exists as regards the width of the chromatin threads. As seen in Figs. 1, 2, 3, 6, and 7, the width of the chromatin thread whether within the framework of the resting nucleus, in the isolated state, or in the form of a metaphase chromosome, is of the same order of magnitude. The metaphase chromosomes of Fig. 4 are thicker and more diffuse and have undoubtedly been swollen by the action of the different staining technique."

Now chromosomes are characterized just by the fact that they vary greatly in width due to different degrees of coiling. This is why metaphase chromosomes are wider, and not because they are "swollen by the action of the different staining technique." Interphase chromosomes very often show variations along their length (heterochromatin—euchromatin) which are characteristic features of specific chromosomes and not artefacts due to fixation as Claude and Potter assume. Such heterochromatic segments are also present in their nuclei. The fact that their isolated chromatin filaments do not show these individual variations in structure would indicate that they are either *de novo* formations (nucleoprotein fibers) or that they have been greatly changed during preparation. The second alternative seems now more probable and we may assume that the threads prepared from leucemic cells are chromosomes, although modified in structure.

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EXPLANATION OF PLATES

PLATE 1

FIGS. 1 and 2. Isolated chromosomes from calf thymus. Aceto-orcein. ×3000.

FIGS. 3 and 4. Isolated chromosomes from calf liver. Aceto-orcein. ×3000.

FIGS. 5 and 6. Isolated chromosomes from carp erythrocytes. Aceto-orcein. ×3000.

FIGS. 7 and 8. Isolated thymus chromosomes, uncoiled with 2.10^{-3} M KCN. Aceto-orcein. ×3000.

FIGS. 9 and 10. Two identical liver chromosomes from two different preparations. This chromosome can be recognized repeatedly in preparations of isolated liver chromosomes. Aceto-orcein. ×3000.

FIGS. 11 and 12. Two identical thymus chromosomes from two different preparations. Aceto-orcein. ×3000.

FIG. 13. Suspension of isolated thymus chromosomes. Aceto-orcein. ×1200.
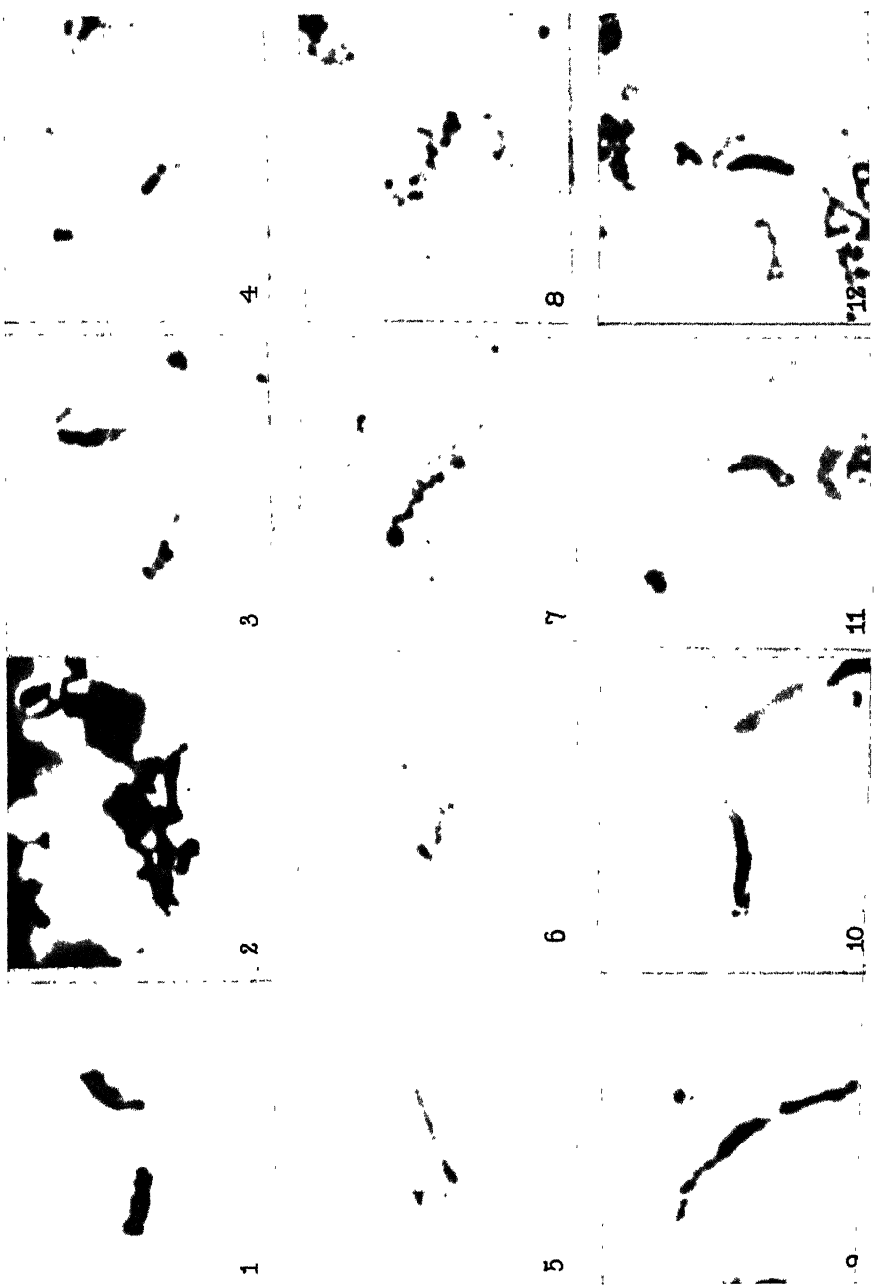
Fig. 13

(Mirsky and Ris: Isolated chromosomes)

THE CHEMICAL COMPOSITION OF ISOLATED CHROMOSOMES

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PLATE 3

The isolated chromosomes, whose chemical composition is to be described in this paper, are derived from the lymphocytes of calf thymus. The composition of these chromosomes has been investigated with reference only to their nucleic acid and protein contents, simply because these are quantitatively the major constituents. The procedure for isolating chromosomes has already been given in a previous paper (1).

By far the larger part of present knowledge of chromosomes comes not from chemistry but from cytology and genetics. Any information concerning the chemistry of chromosomes is accordingly at once placed against a background of chromosome morphology and cytogenetics. In an investigation of the chemistry of isolated chromosomes the chemical basis of chromosome structure will be sought; and in the present investigation this aspect of chromosome chemistry is considered. The results obtained may be briefly summarized: isolated lymphocyte chromosomes can be separated into two parts, of which one (some 90 per cent of the mass of the chromosome) is mainly desoxyribose nucleohistone and the other a coiled, thread-like structure containing non-histone protein combined with relatively small quantities of ribose and desoxyribose nucleic acids; this thread still shows the main characteristics of chromosomes, such as coiling, subdivision into heterochromatic and euchromatic sections, and constrictions; it appears to be imbedded in a mass of nucleohistone; both the thread and the material in which it is imbedded contain nucleoproteins, but of entirely different kinds.

Elementary Composition.—The phosphorus content of isolated lymphocyte chromosome (after extraction with alcohol and ether, to remove lipids) is 3.7 per cent and the nitrogen content 15.6 per cent. It can be shown, in a manner to be described below, that nearly all the phosphorus in such a preparation is present as desoxyribose nucleic acid, which accordingly constitutes close to 37 per cent of the mass of these lipid-free chromosomes.

Histone Content.—Thymus histone has been known for many years, and there has been little doubt that this highly characteristic basic protein is located in the chromosomes of the thymus. That this is in fact true, can be demonstrated by releasing histone from isolated thymus chromosomes. Kos-

sel's procedure for extracting histone was to treat the histone-containing tissue with an excess of dilute hydrochloric or sulfuric acid (2). When an excess of acid is added to isolated chromosomes, histone is released; indeed, little in addition to histone passes into solution. There is no difficulty in identifying as a histone the protein in solution. The basic character of this protein becomes apparent when dilute sodium hydroxide is added to the acid solution, for the protein remains dissolved until the medium becomes definitely basic, maximum precipitation occurring in the neighborhood of pH 10. If the base used is ammonium hydroxide, the protein precipitate obtained does not redissolve when an excess of this weak base is added. Insolubility in an excess of ammonium hydroxide was observed by Kossel at the time he discovered the histones and has ever since been regarded as one of their important characteristics.

Another property of the protein released by acid from isolated chromosomes which serves to identify it as a histone is its non-precipitability by divalent mercury (3). In the presence of dilute sulfuric acid, divalent mercury is known to be a general protein precipitant, acting rapidly if the solution is warmed to 60°C. Under these conditions histones, unlike other proteins, are not precipitated. When sulfuric acid in which a divalent mercury salt is dissolved is added to isolated chromosomes, histone is released and passes into solution.

The amount of histone released by a strong acid from a nucleoprotein complex can be estimated, and it has frequently been supposed that the histone so released represents the total amount present in the nucleoprotein complex. We have estimated the quantities of histone released from isolated chromosomes by acid and also by acid in which divalent mercury is dissolved. Slightly more histone is released by the latter procedure. It might appear as if the amount of histone released did indeed represent the total quantity present in the chromosomes, for the quantities so estimated are reproducible and repeated extraction releases no more histone. We find, however, that much of the histone fails to be released by acid; indeed, a third of the total histone may remain attached to the nucleoprotein complex. If the quantity of non-histone protein in isolated chromosomes or nuclei is calculated by subtracting from the total protein present the amount of histone released by acid, the non-histone content of chromosomes or nuclei will be grossly exaggerated.

Before describing experiments which make possible a more effective fractionation of the proteins present in isolated chromosomes than has heretofore been accomplished, another distinctive property of histones should be mentioned. Histones contain relatively little tryptophane. In some histones such as those of calf liver there is as much as 0.4 per cent tryptophane (and even this can be considered a rather low tryptophane content for a protein); other histones, such as those of the fowl erythrocyte, contain practically no trypto-

phane. The histone released by hydrochloric acid from isolated thymus chromosomes contains 0.14 per cent tryptophane.[1]

Since the generality of proteins contain far more tryptophane, in many cases 1 per cent or more, the low tryptophane content of histones means that finding a considerable quantity of tryptophane in a protein fraction of isolated chromosomes indicates that some other type of protein, a non-histone protein, may be present. In a previous investigation the existence of such a tryptophane-containing protein in chromosomes was recognized (4). Since then a far better procedure has been found for separating the tryptophane-containing protein fraction from the histone fraction, and at the same time the significance of these two protein fractions for the structure of the chromosome has been recognized.

Fractionation of Chromosomes.—Isolated chromosomes are fractionated by placing them in a neutral solution of 1 M sodium chloride. In this medium they at once disperse to form an opalescent, highly viscous suspension. When this striking transformation occurs, one has the impression that perhaps the chromosomes have dissolved, and much material has indeed passed into solution; but when a drop of the viscous opalescent fluid is examined under the microscope, formed structures can still be seen. It might be supposed that the striking increase in viscosity is due to swellling of chromosomes, but centrifugation shows that this is not the case.

To sediment the chromosomes, rapid centrifugation is required because of the exceedingly high viscosity of the medium in which they are suspended. For this purpose we use a centrifuge running at 18,000 to 19,000 R.P.M., with a rotor 30 cm. in diameter, holding 12 tubes, each of 75 cc. capacity. After a run of 1 to 2 hours, the centrifuged material consists of a slightly opalescent but highly viscous supernatant and a scanty, tightly packed sediment. In the supernatant a few chromosomes are visible, but practically all of them are in the sediment. These bodies, which will be referred to as *residual chromosomes*, are resuspended in 1 M NaCl and again sedimented, and this time a far lower speed suffices. After several more washings with 1 M NaCl, no more material is present in the supernatant. The residual chromosomes in 1 M NaCl are stored in the co d.

Under the microscope these threads still look like chromosomes, but they are a good deal smaller. They exhibit longitudinal differentiation into thicker, tightly coiled heterochromatic sections and more loosely coiled euchromatic regions. As in whole chromosomes, constrictions are frequently visible. (Compare Fig. 1 with Figs. 2 to 4.)

[1] More than one kind of histone is present in the thymus. The evidence for this is that the tryptophane content of histone released by hydrochloric acid is less than the tryptophane content of the total thymus histone, 0.4 per cent.

The staining characteristics of residual chromosomes are, when compared with original chromosomes, grossly changed. Before being treated with M NaCl the chromosomes stain intensely with the Feulgen reagent, aceto-orcein, crystal violet, and hematoxylin: (Staining with pyronine-methyl green will be described below). The residual chromosomes stain only faintly with these reagents. Loss of staining properties is not due to some change in physical state of the chromosomes; failure to stain is caused by the removal of material from the chromosomes. The substances responsible for staining are present in the supernatant. M NaCl has, accordingly, separated the chromosomes into two parts: a residue which retains the essential form of the chromosome; and a soluble extract which contains the substances with the characteristic staining properties of chromosomes. The composition of each of these chromosome fractions will now be described.

M NaCl was used by us some time ago to extract a nucleoprotein complex, chromosin, from the cell nucleus (4). At that time the significance for chromosome structure of the different components in the nucleoprotein complex removed from the nucleus was not yet understood.

The Residual Chromosome.—The material that remains insoluble when chromosomes are extracted with M NaCl consists, as has already been said, of microscopic bodies with forms resembling those of the intact chromosomes. After thorough extraction with M NaCl, these residual chromosomes are extracted with alcohol and ether to remove any lipids present, and are then weighed. It is found that they represent only 8 to 10 per cent of the mass of the original (lipid-free) chromosomes from which they were prepared.

By far the larger part of nucleoprotein in the isolated chromosome dissolves in M NaCl, but some nucleoprotein, although of a different kind remains in the residue. The first sign that nucleoprotein may be present is provided by the phosphorus and nitrogen analyses of the lipid-free residue. Phosphorus content varies in different preparations from 1.0 to 1.8 per cent. The nitrogen content is 13.8 per cent. It will soon be seen that all of the phosphorus is in the form of nucleic acid. Presence of protein is indicated by positive tests for a number of amino acids—arginine, cystine, tyrosine, and tryptophane.

Nucleic acid can be removed from a nucleoprotein by heating at 90°C. for 15 minutes in 0.36 M trichloracetic acid, after first treating with cold trichloracetic acid to remove any nucleotides present (5). When this is done with residual chromosomes, no phosphorus is removed by cold trichloracetic acid and all of the phosphorus passes into the solution of hot trichloracetic acid. The absorption spectrum in the ultraviolet of the dissolved material differs slightly from nucleic acid, but the same difference occurs in the absorption spectrum of nucleic acid when it is heated in trichloracetic acid. Considering the phosphorus content of the trichloracetic acid extract the extinction coefficient at the wave length of maximum absorption is very close to that of

nucleic acid of the same phosphorus content. A characteristic of nucleic acid, as compared with simpler nucleotides, is its relatively high molecular weight, rendering it non-diffusible through a cellophane membrane. The material extracted from residual chromosomes by hot trichloracetic acid readily passes through a cellophane membrane, but so also does nucleic acid after the same treatment with trichloracetic acid. It should be noted that a mixture of purines and pyrimidines like those linked together in nucleic acid would have the same absorption spectrum, but if the relative proportions of the four bases were considerably different from the equimolar ratios found in nucleic acid, the difference would alter the absorption spectrum.

Pentose and desoxypentose were estimated in the trichloracetic acid extract by their reactions with orcinol and diphenylamine. The sum of the two sugars was equal to what would be expected if the phosphorus present were all in the form of nucleic acid, part of it as ribose and the rest as desoxyribose nucleic acid. In a nucleic acid the ribose of only the purine nucleosides reacts with orcinol, the ribose of the pyrimidine nucleosides remaining inactive (6, 7); and, similarly, the desoxyribose of only the purine nucleosides reacts with diphenylamine (3). The phosphorus content and extinction coefficient of a nucleic acid are over-all properties of both purine and pyrimidine nucleotides; the orcinol and diphenylamine reactions estimate, on the other hand, constituents of the purine nucleotides only. By combining all these analytical data, the ratio of purines to pyrimidines can be deduced, and in a large number of both pentose and desoxypentose nucleic acids they have been found to be present in a 1:1 ratio. In the trichloracetic acid extract of residual chromosomes, measurements of extinction coefficients and estimates of phosphorus contents and of pentose and desoxypentose contents all indicate that in this material too the purine pyrimidine ratio is close to 1:1. This is strong evidence that the substances extracted from chromosome residues by hot trichloracetic acid are nucleic acids.

Nucleic acid can also be separated from residual chromosomes by allowing them to remain for some time in neutral 0.14 M NaCl. This process will be described below but here it is mentioned because the evidence that it is indeed nucleic acid that is released is more complete than when hot trichloracetic acid is used. In this case the material extracted has precisely the same absorption spectrum as that characteristic of nucleic acid, and furthermore most of it resembles nucleic acid in not diffusing through a cellophane membrane. All of this nucleic acid is of the ribose type, for the desoxyribose nucleic acid present in residual chromosomes is not removed in 0.14 M NaCl. The quantity of pentose determined in this nucleic acid is the same as that found in a sample of ribose nucleic acid of the same phosphorus content prepared from yeast, indicating that in the ribose nucleic acid of residual chromosomes as in that of yeast there is a 1:1 ratio of purine to pyrimidine nucleotides.

The nucleic acid content of residual chromosomes represents only about 4 per cent of the total amount present in whole chromosomes. The rest is extracted with M NaCl. The nucleic acid content of the residual chromosome is mainly of the ribose type, although some desoxyribose nucleic acid is also present. This is in sharp contrast to the distribution of nucleic acids in the chromosome as a whole, in which some 95 per cent of all the nucleic acid is of the desoxyribose type, so that there is some difficulty in detecting that ribose nucleic acid is present. Once the mass of nucleic acid has been removed from chromosomes by extraction with M NaCl, there is no difficulty in detecting ribose nucleic acid in the residues. In different preparations the ribose nucleic acid content of residual chromosomes varies from 7.5 to 14 per cent, and the desoxyribose nucleic acid from 1.5 to 2.6 per cent.

It may be supposed that the small quantity of desoxyribose nucleic acid in the residual chromosomes is merely a "contamination" remaining after removal of nucleohistone with M NaCl. All that can be said at present is that every preparation of residual chromosomes contains some desoxyribose nucleic acid. Most of it is released from the residual chromosomes by desoxyribose nucleodepolymerase.

The staining properties of residual chromosomes can be correlated with what is known of their nucleic acid contents. They are Feulgen-positive, but only faintly so. When one considers that residual chromosomes contain no more than 2.6 per cent of desoxyribose nucleic acid, a positive reaction shows how sensitive the Feulgen technique is. Stained with the pyronine-methyl green mixture of Unna-Pappenheim, residual chromosomes are red, whereas the same dye mixture stains the original isolated chromosomes purple-blue. Brachet has shown that in general the pyronine-methyl green mixture stains desoxyribose nucleoproteins blue-green and ribose nucleoproteins red. The staining of chromosomes and residual chromosomes with pyronine-methyl green is, then, what would be expected, considering their respective nucleic acid contents. The staining of residual chromosomes with pyronine-methyl green shows that the ribose nucleic acid present in a preparation of these bodies is actually in the residue itself and not merely in some other material present in the preparation. This is worth noting because it is known that nucleoli contain ribose nucleic acid and in preparations of isolated chromosomes some nucleoli can indeed be seen. It cannot be said how significant the ribose nucleic acid content of nucleoli is for the over-all determination of ribose nucleic acid in a suspension of residual chromosomes but staining shows that by far the larger part of this nucleic acid is present in the residual chromosomes themselves. It should be noted that by staining with pyronine-methyl green Brachet has already provided evidence that in some chromosomes ribose nucleic acid is present (8).

Even after removing the nucleic acids of residual chromosomes with hot trichloracetic acid or by allowing them to remain in 0.14 M NaCl, these bodies still retain their structure. The protein component of residual chromosomes is, accordingly, responsible for their structure. This protein will be referred to as the *residual protein* of the chromosome. Residual protein represents some 8 per cent of the mass of the isolated lymphocyte chromosome. Histone, on the other hand, accounts for 55 per cent of the mass of these chromosomes. Residual protein and thymus histone differ in numerous other respects: histone contains 18 per cent of nitrogen, residual protein close to 13 per cent: histone is soluble in a $HgSO_4$ — H_2SO_4 mixture, whereas residual protein, like the generality of proteins, is insoluble in the $HgSO_4$ — H_2SO_4 reagent; histone is soluble in water and in dilute acid, but residual protein has so far not been soluble in any medium in which proteins remain intact; residual protein contains 1.36 per cent tryptophane whereas thymus histone contains 0.14 per cent. Investigation of the properties of residual protein has only just begun, but it is already clear that it is quite different from histone, not possessing those properties of histone which distinguish this type of protein from other proteins.

Both histone and at least a part of the residual protein occur as nucleoproteins in the chromosome. Among the numerous differences between these nucleoproteins the nature of the linkage between nucleic acid and protein should be considered. In nucleohistone there is at present no reason to think that there is any more than a salt-like linkage between the two components. In the residual protein it seems likely that nucleic acid and protein are in something more than salt-like combination, for concentrated neutral salt does not dissociate this nucleoprotein, as it does a nucleohistone.

It will be of interest to know which of the enzymes present in chromosomes are located in the residual chromosome. At present it may be said that all of the alkaline phosphatase is located in the residual chromosome.

In a previous investigation of the constituents of chromosomes a non-histone, tryptophane-containing protein was isolated. It seems apparent that this protein is the same as what is now recognized as the protein component of the residual chromosome.

The chromosome fraction soluble in 1 M NaCl; i.e., the supernatant obtained when a suspension of chromosomes in M NaCl is centrifuged at high speed. This solution, it will now be seen, consists largely of nucleohistone.

When the supernatant is diluted with six volumes of water, a beautiful fibrous precipitate forms. This precipitate readily redissolves in M NaCl. Nuclear material with this property has been encountered before (3, 4), and it is now obvious that previous preparations contained some residual chromosomes in addition to nucleohistone. Combining the information we now have with that obtained previously, it is apparent that when a suspension of chromosomes in M NaCl is centrifuged at high speed, residual chromosomes sediment

first, leaving nucleohistone in solution. More prolonged centrifugation (as carried out in previous work) leads to a fractionation of nucleohistone because nucleic acid sediments faster than histone, so that the top layer of fluid after prolonged centrifugation consists almost entirely of histone. One sign of this fractionation is that when the top layer, containing histone, is diluted with six volumes of water, no precipitate forms. A fibrous precipitate appears under these conditions only when both histone and highly polymerized desoxyribose nucleic acid are present. The quantity of precipitate formed from a given volume of the M NaCl supernatant after sedimentation of residual chromosomes is found by washing the precipitate with hot alcohol, ether, and determining the dry weight. The phosphorus content of this material is 4.4 per cent, equivalent to a nucleic acid content of 45 per cent.

Nucleic acid in the M NaCl supernatant can be detected spectrophotometrically. The absorption spectrum of such a solution is compared with that of a nucleic acid solution of the same phosphorus content. The curves are identical down to 2450 A.u.. The divergence at shorter wave lengths is due to presence of histone in the supernatant. The equality of the extinction coefficients at 2600 A.u. shows that the same quantity of nucleic acid is present in both solutions and, therefore, that all of the phosphorus in the M NaCl supernatant is in the form of nucleic acid.

Practically all of this nucleic acid is of the desoxyribose type. This is shown by carrying out the diphenylamine reaction for desoxyribose on the supernatant and on a sample of pure desoxyribose nucleic acid of the same phosphorus content. The same color intensities are obtained. This agreement, as has been explained above, also shows that the purine pyrimidine ratio in the M NaCl supernatant is 1:1, as in nucleic acid itself. The phloroglucinol reaction (9) for ribose shows that a small quantity of ribose nucleic acid is probably present in the M NaCl supernatant.

To estimate the histone content of the M NaCl supernatant, a given quantity is added to six volumes of water, and histone is released by treating the fibrous precipitate so obtained with a $HgSO_4 - H_2SO_4$ mixture. When sodium nitrite is added to histone in this solution a derivative is formed with an absorption band at 3540 A.u.. The histone concentration is determined by measuring the extinction coefficient of 3540 A.u. and comparing it with that given by a solution of known histone content. This is the Millon reaction. A new feature in the procedure that has just been described is that whereas the familiar red pigment produced in the Millon reaction has an absorption maximum at 5000 A.u., another absorption maximum at 3540 A.u. with much higher extinction coefficient has been found.

The histone released and estimated in this way is equal to 47 per cent of the mass of the fibrous precipitate. Since 45 per cent of this precipitate consists of nucleic acid, 8 per cent would still be unaccounted for. This, as will now be

shown, is histone that is not released from nucleic acid by either hydrochloric or sulfuric acid. That some protein still adheres to nucleic acid after treatment with the $HgSO_4 - H_2SO_4$ reagent is apparent from the red color of the precipitate when sodium nitrite is added.

A method is needed for removing and collecting all the protein from the nucleic acid in the M NaCl supernatant. We have already used such a procedure in our previous work (4). Just sufficient sodium hydroxide is added to the supernatant so that no precipitate is formed when a sample is added to six volumes of water. The alkaline supernatant is shaken vigorously or mixed at high speed with a chloroform-octyl alcohol solution, as used by Sevag. When this mixture is centrifuged, three layers are obtained. The top layer consists of a solution of nucleic acid, nearly free of protein, and the middle layer consists of protein, quite free of nucleic acid.

The protein in the middle layer can be collected without loss, washed free of salt, dried, and weighed. It represents 59 per cent of the mass of chromosomes from which it was prepared. This, added to the 37 per cent of nucleic acid present in the chromosomes, accounts for 96 per cent of the total chromosome mass. Practically all of the protein collected in the middle layer is of the histone type.[2] One indication of this is that when the $HgSO_4 - H_2SO_4$ reagent, which precipitates most proteins but dissolves histones, is added to a suspension of this material, almost all of it dissolves, the insoluble residue being insignificant. If the quantity of histone in solution is estimated by measuring the extinction coefficient at 3540 A.u. of the derivative formed by adding sodium nitrite, this is found to be the same as the total quantity of protein in the suspension as determined by dry weight.

Further evidence that nearly all the protein collected in the middle layer is of the histone type is that all of it is basic. The protein collected in the middle layer can be dissolved by adding a little dilute hydrochloric acid. Practically all the protein dissolves at a pH close to 4.0. When sodium hydroxide is added to this solution, no precipitate appears until a pH of over 9 is reached and maximum precipitation occurs between pH 9.3 and 10.0. Of the total protein, 92 per cent precipitates, and this must be considerd to be definitely basic protein. The protein that remains in solution is also basic, because it combines with nucleic acid at pH 8.0 to form a fibrous precipitate.

Instability of Chromosomes.—Both whole isolated chromosomes and residual chromosomes show marked instability under certain conditions. A suspension of residual chromosomes is quite stable at 0° in M NaCl. But when the salt concentration is reduced to 0.14 M (physiological saline) and the pH is adjusted

[2] In recent experiments on the chemical composition of isolated liver chromosomes, we find that a considerable part of the protein collected in this way is of a non-histone type. It is likely that a small quantity of this non-histone type of protein is also present in lymphocyte chromosomes.

to 6.8, a change occurs, slowly in the cold and exceedingly rapidly at room temperature. This change is demonstrated by centrifuging portions of the suspension of residual chromosomes and examining the clear supernatant solution. It contains ribose nucleic acid and a somewhat smaller quantity of protein. In the course of time, several days in the cold, nearly all of the ribose nucleic acid (and none of the desoxyribose nucleic acid) is split off from the residual chromosomes. The long time course of the reaction and the fact that it does not occur, or does so very slowly, in the presence of 2 M NaCl at pH 6.8 make it appear unlikely that a mere acid-base dissociation of nucleic acid takes place, for in the presence of concentrated electrolyte such dissociation would be increased. The change in composition of the residual chromosomes does not affect their microscopic appearance.

Since this change occurs in whole chromosomes, as well as in residual chromosomes, it must take place while chromosomes are being isolated. This means that the quantity of ribose nucleic acid found in residual chromosomes is always less than that present in chromosomes within an intact nucleus. The variations in composition found in different preparations of residual chromosomes are probably due, at least in part, to differences in the quantities of ribose nucleic acid split off while chromosomes are being isolated.

The instability of whole chromosomes is far more striking than is that of residual chromosomes. In saline at a pH of 7.0, and more rapidly at higher pH, chromosomes in a suspension tend to stick to each other. At room temperature stickiness increases with time far more rapidly than in the cold. The change is obvious in a flask of chromosomes, because larger and larger clumps are formed until finally the chromosomes stick together in one gelatinous mass.

Accompanying this gross transformation are finer morphological and chemical changes. In the fluid in which the chromosomes are suspended, protein and nucleic acid appear. Analysis shows that this is largely tryptophane-containing protein and ribose nucleic acid—the constituents of the residual chromosomes These chemical transformation indicate that the substance of the residual chromosome is passing into solution, and this is borne out by microscopic observation. In the course of time fewer and fewer chromosomes can be seen until finally almost none are left. The gelatinous material dissolves in 1 M NaCl, but on high speed centrifugation only a small part of the original mass of residual chromosomes is found; their substance has passed into solution. The nucleohistone in 1 M NaCl appears to be much the same as that formed from a suspension of unchanged chromosomes, and on dilution with six volumes of water it forms the fibrous precipitate characteristic of nucleohistone. Apparently the changes that occur are limited essentially, though not entirely, to the materials of the residual chromosomes, and when their structure undergoes dissolution, no chromosome structure remains. The process of autolysis in the residual chromosome while it still is imbedded in the whole chromosome goes much farther than it does in the separated residual chromosomes.

The autolytic changes that take place in isolated chromosomes are of interest partly because it is possible that the substances discharged from the chromosomes play a part in interactions within the nucleus and in interactions between nucleus and cytoplasm.

Chromosomes Other Than Those Isolated from Lymphocytes.—Some preliminary observations on other chromosomes will be described. Chromosomes isolated from fish (carp) erythrocytes have been studied. Their nucleic acid content, 41 per cent, is somewhat higher than that of lymphocyte chromosomes. In carp erythrocyte chromosomes the residual chromosome represents an even smaller fraction than it does in lymphocyte chromosomes. In mammalian liver chromosomes, on the other hand, the residual chromosome fraction seems to be relatively larger than it is in lymphocyte chromosomes. Residual chromosomes can probably be seen in the nucleus of the trout sperm after treatment with M NaCl, but a quantitative study of them has not yet been made. What appears to be the equivalent of residual chromosomes can be seen in grasshopper spermatocytes and in the salivary gland nuclei of Sciara after treatment with M NaCl.

These observations indicate that residual chromosomes are constituents of many, if not of all, chromosomes. The relative size of this component of a chromosome will to a considerable extent determine its appearance and especially its staining properties. It is also apparent from the examples that were mentioned in the preceding paragraph and from a host of other possibilities that can be culled from the great amount of literature on chromosomes that recognition of the dual nature of the chromosome (consisting as it does of residual chromosome and of nucleohistone) should be of value in understanding the physiological functions of chromosomes. If for example lymphocyte, liver, erythrocyte, and sperm chromosomes differ in their composition, these differences may be correlated with special properties of the cells in which they are located.

Since Heidenhain, cytologists have often distinguished between basichromatin and oxychromatin, a distinction based on reactions towards basic and acid dyes. It appears likely now that these two chromatins are indeed different substances, oxychromatin corresponding to the residual chromosome, basichromatin to nucleohistone. If whole chromosomes are stained with safranin and light green, they stain bright red. Residual chromosomes, however, appear greenish red, taking only little of the basic safranin and more of the acid light green.

SUMMARY

By means of 1 M NaCl isolated lymphocyte chromosomes can be separated into two fractions, each of which contains nucleoprotein. The fraction soluble in M NaCl consists largely of desoxyribose nucleohistone, and constitutes 90 to 92 per cent of the mass of the chromosome. The insoluble residue (the residual

chromosome is a coiled thread containing some 12 to 14 per cent of ribose nucleic and about one-fifth as much desoxyribose nucleic acid; the residual chromosome accounts for 8 to 10 per cent of the mass of the chromosome. The staining of chromosomes—whether by the Feulgen procedure, by hematoxylin, orcein, or by basic dyes such as crystal violet—is due to the nucleohistone fraction which contains about 96 per cent of the nucleic acid of the chromosome. The form of the chromosome is due primarily to the protein thread of the residual chromosome. This thread is the only linear structure of microscopic dimensions in the chromosome that is not readily dispersed. When chromosomes are broken, it must be supposed that a break is made in the protein thread of the residual chromosome. The foregoing provides evidence for considering the residual chromosome to be the basis of the linear order of the genes. This would mean either that the residual chromosome is a structure around which the genes are organized or that the genes form part of its substance.

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EXPLANATION OF PLATE 3

PLATE 3

Magnification of all figures 3000.

FIG. 1. Isolated thymus chromosome. Aceto-orcein.

FIGS. 2 to 4. Thymus residual chromosomes, fixed in acetic-alcohol, stained with pyronin.

1

2

3

4
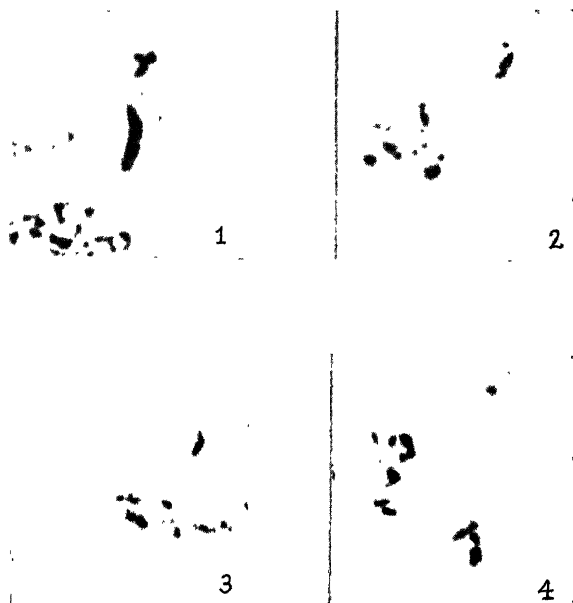
(Mirsky and Ris: Chemical composition of isolated chromosomes)

IDENTIFICATION OF MONO-IODOTYROSINE FROM IODINATED PEPSIN

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In an earlier paper (1) the writer reported the isolation of *l*-mono-iodotyrosine from an alkaline hydrolysate of very slightly iodinated pepsin. Since then Harington and Pitt Rivers (2) have synthesized *l*-mono-iodotyrosine and *dl*-mono-iodotyrosine and a comparison of the properties of their compounds with those reported by us led them to conclude that for the compound from pepsin "the nature remains in doubt."

Differences were noted by them in the following properties:

1. *Specific Optical Rotation.*—The $[\alpha]_D^{\frac{1}{2}\,\text{per cent}}$ of synthetic *l*-mono-iodotyrosine was 4.4 in N·HCl whereas I had obtained 8.8.

2. They found that the *dl*-mono-iodotyrosine crystallized from water with 1 mol of water as revealed by elementary analyses. The writer's analyses agreed with those calculated for a non-hydrated product.

3. In contrast to our positive test they failed to obtain a red color with nitrous acid and ammonia (3).

In view of the previous demonstrations (4–6) of the relationship of pepsin tyrosine and protease activity, and of the possibility that our product from pepsin might be an isomer of 3-iodotyrosine, the isolation from an alkaline hydrolysate of mildly iodinated pepsin was performed as previously described (1). *dl*-mono-iodotyrosine was also synthesized as described by Harington and Pitt Rivers (2). After each had been recrystallized four times their properties were determined and the results compared. The detailed results are reported below but may be summarized here. The specific optical rotation of the freshly isolated product from pepsin was found to be so much less than previously reported that we have some doubts of its being optically active. Nearly all other properties, however, were the same as reported earlier. In addition to the above properties, cross-solubility, cross-partition experiments between immiscible solvents, and the ultraviolet absorption spectra at various pH from which pK were calculated leave little doubt but that the product from pepsin is 3-mono-iodotyrosine. Our synthetic product and that from pepsin both responded positively to the nitrous acid-ammonia color test, but it was possible to free the synthetic product of its color-giving component.

From the analyses of successive extractions with butyl alcohol of an acid hydrolysate of iodinated pepsin, a calculation was made of the quantity

of mono-iodotyrosine originally present. This calculation indicated that 73 per cent of the total iodine was mono-iodotyrosine and the bulk of the remainder of iodine behaved with respect to partition experiments like di-iodotyrosine. This is evidence that only tyrosine in pepsin and not some other amino acid or structure reacts with iodine.

<div align="center">RESULTS</div>

Optical Rotation.—Harington and Pitt Rivers (2) expressed surprise, not wholly unwarranted, that our product possessed optical activity after extensive alkaline hydrolysis. However, Dakin (7) has shown that "end" amino acids are much more resistant to racemization during alkaline hydrolysis, and it was felt that since only one or possibly two tyrosine residues in each pepsin molecule was iodinated, it might be an end amino acid of a side chain and hence resist the alkaline racemization.

The earlier report contained an $[\alpha]_D^{4\,\text{per cent HCl}} = 8.8$, whereas Harington and Pitt Rivers found 4.4. Our present results using 35 mg. of five times recrystallized material dissolved in 1.5 ml. N HCl showed a negative rotation of 0.03° or $[\alpha] = 1.3$. This result casts considerable doubt on the earlier value and suggests that the product may be optically inactive. Unfortunately, insufficient material prevented an unequivocal answer on this point.

Water of Crystallization or Combination.—Ludwig and von Mützenbacher (8), studying mono-iodotyrosine isolated from iodinated casein, and Harington and Pitt Rivers with their synthetic *dl*-iodotyrosine, note that the crystals contained 1 mol of water per mol amino acid. Whereas Ludwig and von Mützenbacher dried their product at 100° *in vacuuo* for 20 hours and made their determination by means of the Karl Fischer reagent, Harington and Pitt Rivers apparently relied on the elementary analyses. The latter workers state that the water could not be removed without decomposition and loss of iodine.

The elementary analyses of our previously isolated product showed no evidence of any water of combination. This was confirmed in the present results shown in Table I. In this respect the product from pepsin resembles Harington's optically active *l*-mono-iodotyrosine which does not contain water of combination.

When our synthetic *dl*-mono-iodotyrosine was tested for water content using the Karl Fischer reagent in a micro apparatus (9), less than 1 mol of water per mol amino acid was found. Using varying amounts of iodoamino acid dried either over drierite, or at 60° *in vacuuo* overnight and varying considerably the time it was allowed to stand in contact with the absolute methanol, we obtained 1.4 to 1.8 per cent water while 1 mol of water calls for 5.5 per cent.

Ultraviolet Absorption Spectra.—The absorption spectrum of a resonating compound such as the one under discussion is a highly specific property and

as such is very useful for identification purposes. From the work on somewhat similar compounds (10) it was expected that ortho- and meta-iodotyrosines would have detectably different absorption spectra in the ultraviolet region.

If the iodotyrosine from pepsin was an isomer of 3-iodotyrosine, then its

TABLE I

Elementary Analyses

Material	Drying process	Dry weight				
		C	H	N	I	Ash
		per cent	*per cent*	*per cent*	*per cent*	*per cent*
$C_9H_{10}O_3NI$ (calculated)		36.1	3.7	4.6	41.4	
$C_9H_{10}O_3NI \cdot H_2O$ (calculated)		33.2	3.7	4.3	39.1	
3-Iodo *l*-tyrosine (Harington and Pitt Rivers)		35.6	3.8	4.8	41.1	
3-Iodo *dl*-tyrosine (Harington and Pitt Rivers)		33.7	3.9	4.4	38.8	
4 times crystallized 3-iodo-*dl*-tyrosine (Herriott) Repeat analysis*		37.0 36.6	3.8 3.5	4.8 5.0	38.9 38.3	0.4 0.17
" "	65° C. *in vacuuo* for 18 hrs.				39.2 38.8	
5 times crystallized material isolated from iodinated pepsin*		35.3	3.5	4.9	40.8	0.95
" "	65° C. *in vacuuo* for 18 hrs.				40.8 41.0	
Crystalline iodotyrosine from pepsin reported in 1941				4.5	42.0	

* Analyses made by A. Elek, Rockefeller Institute, micro analyst.

absorption spectrum would be expected to be different from that of the synthetic product at some pH, particularly in the region where the phenol group of one is 50 per cent ionized.

Fig. 1 contains the absorption curves of equivalent amounts of tyrosine, mono-iodotyrosine (synthetic and from pepsin), and di-iodotyrosine at pH 1.1, 7.6, and 13. Also included are the curves of the two mono-iodotyrosines at pH 8.12 where the ionization is very close to 50 per cent.

It may be seen that the spectra of the product from pepsin are very similar to those of synthetic 3-iodo *dl*-tyrosine and are very different from those of either tyrosine or di-iodotyrosine. The difference of 10 to 15 per cent in the mono-iodotyrosine curves determined at pH 13 is probably due to differences in concentrations through some error, for in other experiments no such differences were observed.
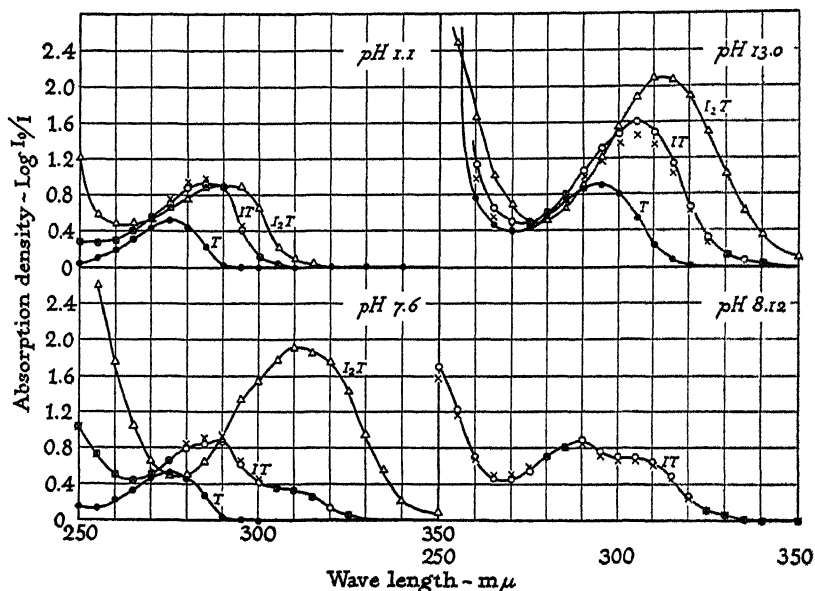
FIG. 1. Ultraviolet absorption spectra of equivalent amounts (5.0 μg N/ml.) of tyrosine (T) indicated by ●, mono-iodotyrosine (IT) from pepsin marked ○, synthetic mono-iodotyrosine marked X, and di-iodotyrosine (I₂T) marked △, in solutions of different pH.

pK.—Determinations of the ionization constants of phenols by observing changes in the absorption spectra with changes in pH were described by Stenstrom and Goldsmith (11). Other have found this method useful (12, 13). Using this method the *pK* of the phenolic group of mono-iodotyrosine has been calculated. A value of $pK = 8.2$ was obtained for both the synthetic derivative and the product from pepsin.

Solubility Experiment.—A solubility curve of five times crystallized synthetic 3-iodo-*dl*-tyrosine in water at 9°C. is shown in Fig. 2. The solid line is the expected curve of a single substance. The experimental points fall close to this curve.

It would have been desirable to compare the complete solubility curves of

both the mono-iodotyrosines, but owing to the small amount of material from pepsin a slightly different experiment was performed. The results are shown in Table II. In general, if two substances are different, a saturated solution of one material will be expected to dissolve some of the second substance. This concept has served as the basis of the present experiments.
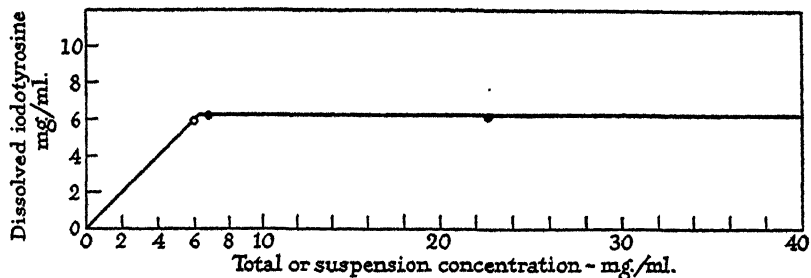
FIG. 2. A solubility curve of synthetic *dl*-mono-iodotyrosine in water at 9°C. The sample indicated by the open circle O contained no solid phase after equilibration. Samples indicated by closed circles ● contained excess solid phase at equilibrium.

TABLE II

	Dissolved iodotyrosine	
	From iodine analyses	From spectrophotometric analyses
	mg./ml.	*mg./ml.*
Original saturated solution............................	6.1	
(*a*) After equilibrating with iodo material from pepsin...	6.2	6.4
(*b*) After equilibrating with synthetic iodotyrosine......	5.8	6.2

EXPERIMENTAL PROCEDURE

Water was saturated by stirring overnight with a large excess of five times crystallized synthetic *dl*-iodotyrosine after which the solid phase was separated by filtration. To 1 ml. aliquots of the filtrate were added 3 mg. of five times crystallized (*a*) iodotyrosine from pepsin, (*b*) synthetic iodotyrosine. These suspensions were then equilibrated for 24 hours at 10°C. by rotating about its short axis a small test tube containing the suspension plus a glass bead. After equilibration and centrifugation the concentration of material in the supernatants was determined from iodine and spectrophotometric analyses at pH 7.6 and wave lengths from 285 to 310 mμ.

The iodine and spectrophotometric analyses in Table II indicate that the iodo product from pepsin did not dissolve. Thus no difference in preparations could

be detected by this extremely sensitive test. This experiment also suggests, though by itself does not prove, that the material from pepsin is probably not optically active, since the solubilities of optically active enantiomorphs are often qualitatively independent of the solubility of the racemic compound.

Partition or Distribution Coefficient.—The results of distribution experiments on the synthetic iodotyrosine and the iodo product from pepsin are shown in Table III. The solvents were dilute acid (pH 2) and butyl alcohol. Mixed or cross-distribution experiments were also performed. After equilibration the aqueous phases of each system were reequilibrated but with the butyl

TABLE III

Experimental Procedure

To 5 ml. of the solutions containing 1 mg./ml. of the iodo products in two separate tubes was added 0.15 ml. of 0.35 N HCl to pH 2.1 followed by 5 ml. of reagent grade normal butyl alcohol. These were shaken hard and after separation was complete aliquots of butyl alcohol layers were analyzed for iodine. The upper alcohol volume was 4.45 ml. while the aqueous layer was 5.7 ml. 3.5 ml. of the butyl alcohol layers were then drawn off and superimposed on the aqueous layer of the opposite system, and after reequilibration the iodine analyses were again made on the butyl alcohol of the two systems. The concentration in the aqueous layers was calculated.

No.	System	Mg. iodine/ml.		Partition or distribution coefficient* $[I]_B/[I]_{H_2O}$
		Butyl alcohol	Aqueous	
1	Iodotyrosine (from pepsin)	0.166	0.22	0.75
2	3-iodotyrosine (synthetic)	0.166	0.22	0.75
3	Aqueous (1) and butyl alcohol (2)	0.154	0.23	0.67
4	Aqueous (2) and butyl alcohol (1)	0.162	0.225	0.71

* In experiments employing dilute H_2SO_4 at pH 3, in place of the above HCl at pH 2.1, the coefficient was 0.6 − 0.65 for both iodo products.

alcohol of the other system. After reequilibration the concentration of iodine in the various solutions was determined and the coefficients calculated.

The results of these experiments shown in Table III demonstrate that the distribution coefficient is the same for all these various systems. There is, therefore, no indication from these experiments that in these two compounds the position of the iodine is different.

Nitrous Acid—Ammonia Color Test.—Harington and Pitt Rivers (2) were unable to produce a red (*i.e.* positive) color when examining mono-iodotyrosine with the nitrous acid—ammonia color reaction (3). The writer obtained a positive color test with nearly all samples of four or five times crystallized synthetic or peptic products. In the case of the synthetic, however, it was found that upon fractional crystallization of an already five times crystallized preparation, the first fraction gave a strong color reaction, the second less, and

the mother liquor no color reaction, the same quantity of material being analyzed in each instance. It is clear in this case that the color arose from an impurity.

It is possible that traces of di-iodotyrosine carried along with the mono-iodo derivative from pepsin account for the positive reaction of the latter. Unfortunately, when it was discovered that the synthetic product gave a negative reaction, we no longer had any of the amino acid from pepsin to fractionate and examine in the above manner.

Amount of Mono-Iodotyrosine in the Hydrolysate of Iodinated Pepsin.—In our previous communication (1) separation of the iodine-containing component was effected by precipitation with lead acetate, which involved very little loss of iodine, followed by successive extractions with butyl alcohol. A calculation of the distribution coefficient for the iodine component after each extraction (solutions 14 to 21 in Table II of a previous paper (1)) shows that there was a gradual drift in the coefficient from 1.0 to 0.6. This indicates the presence of more than one iodine component. The coefficients for the last two extractions were 0.62 and 0.61, or virtually identical with the value obtained for crystalline synthetic mono-iodotyrosine (see footnote in Table III) as well as that for the crystalline iodo product from pepsin obtained under the above conditions. Using the value of 0.6 for the coefficient, the quantity of mono-iodotyrosine in each of the preceding fractions of this extraction series was then calculated. The sum of these quantities totaled 73 per cent of the total iodine at the start. This quantity was confirmed in the present work in which no preliminary purification procedures were involved. The hydrolysate after removal of the barium hydroxide was merely titrated to pH 3 and extracted repeatedly.

A calculation was also made of the coefficient of the 27 per cent remaining iodine. A somewhat variable value in the neighborhood of 2.5 was obtained, which is close enough to the value of 2.1 for di-iodotyrosine to be very suggestive—particularly since di-iodotyrosine would from the nature of the iodination reaction be expected to be present. Thus with virtually all the iodine accounted for, it seems highly improbable that any structure other than tyrosine in pepsin is iodinated under the conditions specified.

EXPERIMENTAL DETAILS

Preparation of Iodotyrosine from Iodinated Pepsin.—Two lots of crystalline pepsin were iodinated to a slight degree (0.7 per cent or approximately 2 atoms of iodine per mol of pepsin) and the iodine compound isolated in one instance by the method previously described. In the second instance the butyl alcohol extraction preceded the precipitation with lead acetate. Four to five crystallizations were necessary to free the material of a brownish colored contaminant and other materials, to a point where the absorption spectra of the crystals and mother liquor were identical.

Preparation of Synthetic dl-Mono-Iodotyrosine.—Synthesis of this product was per-

formed according to procedure of Harington and Pitt Rivers (2). One change was made in the final steps of the preparation which appeared to improve the yield. After decomposition of the diazonium salt by boiling concentrated KI, 45 gm. (0.15 moles) of Ba $(OH)_2 \cdot 2 H_2O$ were dissolved to neutralize the H_2SO_4. Upon filtering, washing the residue, and evaporating the wash water and filtrate *in vacuo*, a nearly colorless product was obtained. When this neutralization was omitted appreciable quantities of dark material contaminated the crystals. Our final product was four times recrystallized from hot water at pH 5 to 6.

The absorption spectra were determined with the aid of a Beckmann D.U. ultraviolet spectrophotometer standardized at 656 mμ according to the manufacturer's directions and in the ultraviolet against 0.02 per cent reagent grade benzene in isooctane.

The writer was assisted in this work by Mrs. Helen F. Dunk.

SUMMARY

Isolation of mono-iodotyrosine from slightly iodinated pepsin has been repeated and the properties of the product compared with those of synthetic *dl*-3-iodotyrosine. Ultraviolet absorption spectra, *pK* values of the phenol group, solubility measurements, and partition coefficients were so nearly identical for the two materials that there is now no reason to doubt that the product from pepsin is 3-iodotyrosine.

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THE RELATION OF ENZYMATIC ADAPTATION TO THE METABOLISM OF ENDOGENOUS AND EXOGENOUS SUBSTRATES*

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INTRODUCTION

Resting suspensions of yeast cells can acquire the enzymatic apparatus necessary to ferment previous unutilizable substrates. The acquisition of this new enzymatic property follows incubation of the cells with the substrate under appropriate conditions. It has been established (1, 2) that this capacity to form the appropriate enzyme under substrate stimulation is a gene-controlled character and may be inherited in a Mendelian fashion. From the existence of this phenomenon of enzymatic adaptation, it is apparent that the possession by a cell of the appropriate gene does not *per se* guarantee that the corresponding enzyme will be found. Substrate plays a critical rôle in evoking the complete enzymatic potentiality inherent in the genetic constitution. Other implications of this phenomenon for the mechanism whereby genes may control enzymatic constitution have been discussed elsewhere (3–5).

Recent experiments (6) on galactose adaptation have established that the modification which a cell undergoes to be able to ferment galactose involves a change in the apoenzymatic or protein moiety of the enzyme system. The coenzymatic portion of a galactose-adapted cell was found to be functionally identical with that of an unadapted one.

The experimental procedure used in studying the phenomenon of adaptation in yeast usually involves placing the cell in contact with the adaptive substrate as the sole external source of carbon and energy. From the point of view of the physiology of enzymatic adaptation, it becomes of extreme interest to determine the energy source required by the cell to make the appropriate changes in its enzymatic constitution. It has been noted (7–9, 15) that the presence of oxygen greatly stimulates the adaptive process, and in some cases is essential if it is to take place. Presumably, then, some type of aerobic metabolism is the primary source of energy in these instances. It was subsequently demonstrated for galactose adaptation (9) that this requirement for oxygen was confined to the initial period of adaptation. If an adequate amount of enzyme is built up by previous aerobic incubation, subsequent anaerobic contact with

substrate will lead to the formation of more enzyme. This was interpreted to indicate that sufficient enzyme had been formed in the presence of oxygen to permit the cell to utilize the adaptive substrate anaerobically as a source of energy for the formation of more enzyme.

The solution to the problem of why aerobiosis is generally required to initiate the process was answered in large part by the finding (10, 11) that intact yeast cells cannot utilize their carbohydrate reserves anaerobically. These reserves can, however, be readily oxidized aerobically by a pathway which invariably results in an R.Q. of unity or slightly less with no evidence of aerobic fermentation. Under these circumstances it is not surprising to find that adaptation is difficult when initiated under anaerobic conditions, since neither the exogenous adaptive substrate nor the endogenous reserves are utilizable as sources of energy. Aerobically, however, the cells can metabolize the endogenous carbohydrate and use the resulting energy to form the adaptive enzyme.

The suggestion that the aerobic endogenous respiration is the main source of energy for the adaptation to galactose and maltose fermentation makes it desirable to obtain more information on the relation between the level of the endogenous respiration and adaptability. It would also be desirable to know whether it is not possible to replace completely the aerobic endogenous metabolism as an initiator of adaptation by supplying a suitable substrate which could be utilized as a source of energy under anaerobic conditions.

It is the purpose of this paper to discuss results pertinent to these and related questions. The investigations described here were carried out with several different strains of yeast. This was done in an attempt to survey the extent of the range in the physiological behavior with respect to the properties being investigated. Not all of these results need be reported in detail. In each particular instance the data on the most representative strain will be discussed, and, wherever deviations from this norm are sufficiently great to warrant it, they will be noted.

Materials and Methods

(a) *Yeast Strains.*—All strains employed were diploid, as evidenced by their ability to sporulate and to produce viable ascospores. Four of these (LK2G12, 812, A1, K1) are representatives of *S. cerevisiae*. The fifth (C) stems from *S. carlsbergensis*. All five can adapt to ferment galactose directly, without the intervention of cell division.

(b) *Media.*—The culture medium was made by adding the following to 1 liter of water: 2 gm. of autolyzed yeast extract powder, 5 gm. of bacto peptone, 1 gm. $(NH_4)_2SO_4$, 2 gm. of KH_2PO_4, 0.25 gm. of $MgSO_4$, 0.13 gm. of $CaCl_2$, 7 cc. of 50 per cent sodium lactate, and 60 gm. of dextrose. After being brought to a boil, the mixture is cleared by filtration and dispensed into 250 cc. Erlenmeyer flasks in 77 cc. quantities for sterilization and use.

(c) *Methods of Handling Cultures.*—One of the problems always encountered in making physiological studies of yeast strains is the danger of introducing genetic variants. With diploid cultures this is reduced to a minimum, providing sporulation

can be suppressed. It was found that maintenance of high carbohydrate concentrations and frequent transfers reduces sporulation to an undetectable level. Stock cultures of the strains used in the present study were carried in the 6 per cent dextrose medium described above and were transferred every 48 hours. All cultures were incubated at 29°C.

(d) *Manometric Measurements.*—All measurements were done at 30.2°C., and the Warburg vessels were shaken at a rate of 110 complete oscillations per minute through a 7 cm. arc. The anaerobic CO_2 production was determined by replacing the air with nitrogen. Inaccuracies due to retention of CO_2 were reduced by using M/15 KH_2PO_4 as the suspending medium.

(e) *Standard Suspensions.*—To facilitate the rapid preparation of suspensions of the desired density, a photoelectric colorimeter was calibrated so that readings could be interpreted in terms of milligrams of dry weight per cubic centimeter of suspensions. The yeast cells were centrifuged away from the culture medium in 50 cc. tubes at approximately 2,500 R.P.M. for 7 minutes. After allowing the medium to drain out, M/15 KH_2PO_4 was carefully poured down the side so as not to disturb the yeast, and was then poured out and allowed to drain. This served to wash the medium from the sides of the tubes. The cells were then resuspended and centrifuged. This procedure was repeated once more. After this the washed cells were resuspended in M/15 phosphate and adjusted to the desired density. Suspensions prepared in this way contained no detectable fermentable substrate.

(f) *Dry Weights.*—Five or 10 cc. portions of the experimental suspensions were pipetted into absolute alcohol, contained in Jena 164 glass filters. The yeast cells were then separated from the alcohol by suction and washed with several volumes of 30 per cent alcohol. The weighing vessels were allowed to dry in an air oven at 70°C. and then removed to a desiccator and allowed to stand until they came to constant weight.

(g) *Physiological State of Cells and Sterility of Suspensions.*—At the end of every manometric experiment, microscopic examinations of samples from the suspensions were made. The methylene blue technique was utilized to determine the number of dead cells. The number of cells taking up the dye was always less than 1 in 500 in all experiments reported. In all experiments extending over 12 hours, aseptic precautions were employed. No bacterial growth was noted in any of the experiments reported, as tested either by direct examination of stained smears or by streak plates made from samples of the suspensions.

(h) *Carbohydrate Source.*—Reagent grade glucose was used. Difco's purified galactose was further treated according to a method described by Stephenson and Yudkin (12) to remove contaminating fermentable sugars. This latter method was modified to include two recrystallizations from 70 per cent alcohol of the treated galactose. Merck's purified maltose was employed.

EXPERIMENTAL RESULTS

(a) Aerobic Adaptation

To establish the relation between the endogenous metabolism and adaptation, experiments were performed which compared adaptability when the substrate was added at different levels of the endogenous respiration. The

time in hours required to arrive at a $Q_{CO_2}^{N_2}$ of 50 or over, subsequent to the addition of substrate, was taken as a measure of adaptability, and is called the adaptation time. To allow for a more continuous observation of the adaptive process, the appearance and increase of aerobic fermentation was followed by the usual two-cup method (13). This method permits R.Q. determinations directly from the manometric readings. Preliminary experiments were performed with each strain employed to determine the R.Q. which under the conditions of the experiment corresponded to a $Q_{CO_2}^{N_2}$ of about 50. This was checked during the experiment by flushing the KOH-free cup of each pair of vessels and measuring the $Q_{CO_2}^{N_2}$ when the proper R.Q. was reached. This method of measuring adaptation times, while less accurate, is more convenient for the purposes of the present experiment than the direct one of making $Q_{CO_2}^{N_2}$ determinant on aliquots at specified intervals.

The experiments were performed by allowing suspensions of the culture in M/15 KH_2PO_4 to dissimilate while shaking in the Warburg cups. Readings were taken at 10 to 20 minute intervals, and, when the desired respiratory rate level was reached, the adaptive substrate in the side-arm was tipped into the main compartment and the resulting behavior followed. R.Q. calculations were made at 20 minute intervals.

The results usually obtained in such an experiment are exemplified in Fig. 1. For purposes of comparison the time course of the endogenous respiratory levels is also reproduced. It is evident that the adaptation times remain quite constant during the period of steady endogenous respiration. As the latter enters its period of decline, there is a rise from about 3 to $6\frac{1}{2}$ hours in the adaptation time. This longer lag period is then maintained for over $2\frac{1}{2}$ hours, during which period the endogenous CO_2 falls from 12.5 to about 2. Subsequent to this the adaptation time rises sharply, and never consistently levels off.

While quantitative variations are observed when the same experiment is done with other strains, fundamentally they all exhibit more or less the same behavior. The only difference which may be mentioned is that, with two of the strains examined (812 and LK2G12), the second plateau observed in the present instance between 180 and 340 minutes is absent. The adaptation time starts to rise as soon as the endogenous respiratory rate begins to decline and does not level off subsequently.

The general inverse relation between adaptation time and the level of the endogenous respiration observed in Fig. 1 is what would be expected if the oxidation of the carbohydrate reserves supplied the necessary energy. This inference is supported by experiments on the effect of restoring the depleted reserves. This was accomplished by adding glucose to portions of suspensions which had arrived at low endogenous respiration rates. After incubation for 2 hours at 30°C. in 3 per cent glucose, the cells were centrifuged and washed

in the cold with chilled buffer, and resuspended in M/15 KH₂PO₄ to the same density. Their adaptation times and endogenous respiratory rates were then measured. Results obtained in such experiments are given in Table I, in which are recorded respiratory rates and adaptation times before and after the glucose incubation. In every instance, restoration of the carbohydrate reserves and consequent elevation of the respiratory rate was paralleled by a corresponding decrease in adaptation time. Controls which were treated in

Galactose Adaptation
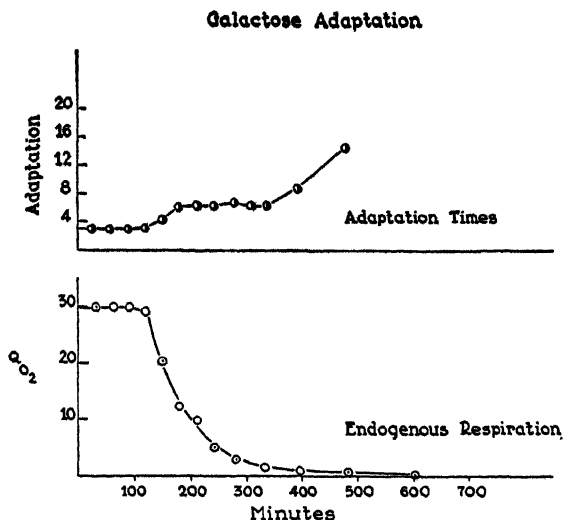
FIG. 1. The relation between aerobic galactose adaptation and the rate of endogenous respiration. The ordinate of the upper curve is the adaptation time, defined as the time in hours required for the suspension, when placed in galactose, to attain a $Q_{CO_2}^{N_2}$ of 50 on galactose (see text for details of method for determining $Q_{CO_2}^{N_2}$ values). The corresponding ordinate on the lower curve is the Q_{O_2} of the endogenous respiration (in microlitres per mg. dry yeast per hour) of the same suspension at the time when it was first exposed to galactose.

exactly the same way, except that no glucose was put in during the 2 hour incubation period, showed no such effect. Analogous findings have been reported (29) in the case of maltose adaptation, where relatively short pretreatment with glucose resulted in marked decreases in adaptation times. It is worth noting that, if the data of Table I are plotted as adaptation time against Q_{O_2}, the points before and after treatment appear to lie on the same curve, indicating that is is the rate of metabolism which is decisive in determining the ease with which adaptation takes place.

While there is little doubt, in view of these experiments, as well as of Fig. 1, that vigorous endogenous respiration shortens adaptation, it is evident that

some other factors are involved. It will be seen from Fig. 1 that adaptation takes place even when most of the oxidizable reserves are gone. This capacity of cells to adapt even when their endogenous Q_{O_2} has sunk to barely measurable levels has been noted with every yeast strain examined. The adaptation is slow, it is true, but nevertheless it does take place; and one is forced to conclude either that adaptation requires little or no energy or that a source of energy exists other than the endogenous respiration. This apparent paradox was solved by the discovery (14) that under aerobic conditions the adaptive substrate can be oxidized before the cells are adapted to ferment it. Hence it

TABLE I

Effect of Reactivating the Endogenous Respiration on Adaptation Time to Galactose

Q_{O_2} is defined as microlitres of O_2 consumed per mg. dry weight of cells per hour. $Q_{CO_2}^{O_2}$ and $Q_{CO_2}^{N_2}$ are defined analogously; the superscripts O_2 and N_2 refer to the gas phases used.

Adaptation time is defined as the time in hours required to reach a $Q_{CO_2}^{N_2}$ of 50 subsequent to addition of adaptive substrate.

Strain	Before treatment		After treatment	
	Q_{O_2}	Adaptation time	Q_{O_2}	Adaptation time
		hrs.		*hrs.*
LK2G12	1.3	12.2	26.1	4.2
	5.1	8.5	29.2	3.1
	0.5	>18*	18.4	7.6
812	7.6	7.0	25.8	2.4
	2.3	8.6	20.2	3.2
	0.4	16.3	15.3	6.1

* The > sign here and in the subsequent tables signifies that the experiment was terminated after this number of hours.

can supply the energy required to form the adaptive fermentative system. The detailed behavior and properties of this preadaptive oxidative system are discussed more completely in the succeeding paper.

(b) Anaerobic Adaptation without Exogenous Substrate

It was found in a previous investigation (9) that some strains possessed the capacity to adapt to galactose under completely anaerobic conditions. The anaerobic adaptation is, however, much slower than the one reached in a corresponding culture under aerobic conditions. This anaerobic adaptability could not be referred to any observable difference in either the aerobic or anaerobic endogenous metabolism. It seemed likely, therefore, that the ability to initiate the adaptation anaerobically could be due to the storage of energy-rich compounds whose breakdown could yield energy for the formation of

sufficient galactozymase to carry the synthesis farther. The difference between those strains capable of initiating adaptation anaerobically and those which cannot would, from this point of view, reduce to a quantitative difference in ability to retain such compounds. This conclusion is supported by the fact that all strains tested, including those that cannot start the adaptive process anaerobically, are able to continue the adaptation under anaerobic conditions once they receive a sufficient start.

Further evidence on this question was sought by examining the relation between anaerobic adaptability and the physiological state of the cultures. The previous (9) experiments referred to were all performed on 48 hour cultures. It seemed desirable to extend the examination to younger cultures. The cellular content of the necessary energy-rich compounds which could be used to initiate the adaptation would probably vary with the physiological condition of the cell, being presumably higher in young and actively growing cultures. Table II summarizes experiments of this nature on five of the strains tested. In all cases, 24 hour or younger cultures possessed anaerobic adaptability with respect to both galactose and maltose fermentation. This is true also for the two cultures previously reported as unadaptable anaerobically on the basis of the examination of a 48 hour culture. Cultures older than 24 hours begin to show marked increases in adaptation time, and in some cases anaerobic adaptability is lost completely. It will be noted further that, whereas anaerobic adaptation time is extremely sensitive to the age of the culture, within the period examined the aerobic adaptation time shows very little difference. This is true for both maltose and galactose adaptation. This comparatively greater sensitivity of the anaerobic adaptation time would be expected if the initiation of the adaptive process does depend on the already formed energy-rich compounds the cell happens to possess at the time anaerobiosis is instituted. Aerobic adaptation, on the other hand, would not be as sensitive, since the necessary compounds could be formed during the course of the respiration.

It might perhaps be supposed that the endogenous respiration would result in the storage of energy-rich compounds which might be utilizable subsequently under *anaerobic* conditions for the purpose of adaptation. To test this possibility, the anaerobic adaptation times of dissimilated and undissimilated cultures were examined. The period of the dissimilation was kept within the constant-rate period of the endogenous respiration. Table III summarizes the data obtained on two strains with respect to galactose and maltose adaptation. It is seen that in every instance anaerobic adaptation time is increased by dissimilation, rather than decreased. For purposes of comparison the measurements on the corresponding aerobic adaptation times are included. Here, as before, we find no observable effect of the dissimilation periods. It must be concluded either that endogenous respiration does not lead to a net increase of energy-rich compounds, or that the compounds formed under these

conditions are not particularly useful in aiding the cell to adapt under *anaerobic* conditions.

TABLE II

The Effect of Age on Anaerobic Adaptation

Adaptation time is time in hours required to reach a $Q_{CO_2}^{N_2}$ of 50.   In all cases the cells were in contact with 3 per cent of the substrate during the adaptation and the determination of the $Q_{CO_2}^{N_2}$.

Strain	Substrate	Age	Anaerobic adaptation time	Aerobic adaptation time
		hrs.	*hrs.*	*hrs.*
C1	Galactose	18	5.1	1.9
		24	4.9	2.2
		36	7.8	2.4
		48	37	2.3
LK2G12	Galactose	18	10.3	2.6
		24	20.4	2.8
		36	>48	3.0
		48	>48	3.0
812	Galactose	18	8.4	1.8
		24	14.2	2.0
		36	>48	2.0
		48	>48	2.2
A1	Galactose	18	6.5	1.4
		24	—	1.6
		36	10.3	1.8
		48	40.1	1.7
K1	Galactose	18	5.8	2.0
		24	6.4	2.4
		36	10.5	2.1
		48	36	2.6
K1	Maltose	36	3.8	1.5
		48	6.2	1.5
		54	>24	1.9
A1	Maltose	36	4.2	1.8
		48	7.6	1.8
		54	>18	2.4

It should be remembered that this same respiratory metabolism is extremely effective in inducing subsequent anaerobic adaptation *providing the adaptive*

substrate is present during the metabolism. This "directive" effect of substrate on the energy-yielding mechanism of the cells is a characteristic of enzymatic adaptation which is observed also under anaerobic conditions.

(c) Anaerobic Adaptation with Exogenous Substrate

The explanation offered for the relative inability of cells in contact only with adaptive substrate to adapt anaerobically is based on the assumption that neither the internal nor the external substrates are readily available as sources of energy for synthetic activity. This would imply that anaerobic

TABLE III

The Effect of Dissimilation on Adaptation Times

Adaptation time is time in hours required to reach a $Q_{CO_2}^{N_2}$ of 50. In all cases the cells were in contact with 3 per cent of the substrate during the adaptation and the determination of the $Q_{CO_2}^{N_2}$.

Strain	Substrate	Period of dissimilation	Anaerobic adaptation time	Aerobic adaptation time
		hrs.	*hrs.*	*hrs.*
K1	Galactose	0	5.3	2.2
		1	>24	2.3
	Maltose	0	3.3	1.5
		1	10.6	1.3
		2	>24	1.7
A1	Galactose	0	6.4	1.5
		1	>48	1.8
	Maltose	0	5	2.0
		1	>24	1.8

adaption could occur if some substrate were supplied which could be utilized under these conditions as a source of energy. Accordingly, the effects of adding small amounts of glucose to cells anaerobically adapting to the fermentation of galactose and maltose were examined. Fig. 2 gives some representative results obtained with galactose adaptation. In these experiments the galactose (final concentration of 3 per cent) and the stated amounts of glucose were added simultaneously from the same side-arm after anaerobiosis and thermal equilibration had been established. The first burst of activity seen is the fermentation of the glucose added. Once this period is over, the galactose fermentation becomes observable.

For the purpose of comparison we may confine our attention to those portions of the curve above the lines corresponding to 100 per cent recovery of the

glucose added.   Any CO_2 released in excess of these amounts must originate from the fermentation of galactose.   It is clear from a comparison of rates beyond 120 minutes that in all cases, including the one where only 0.8 mg. was added, the presence of glucose enables these cells to form galactozymase far

FIG. 2. The facilitation of anaerobic galactose adaptation by small amounts of glucose.   (A) 4 mg. of added glucose; (B) 1 mg. glucose; (C) 0.8 mg. glucose; (D) 1 mg. glucose, a different yeast strain, showing strain difference in response to glucose effect; (E) no added glucose.   Horizontal lines indicate theoretical amount of CO_2 corresponding to amount of glucose added.

in excess of that attained in the control.   The experiment in which 4 mg. of glucose was added shows evidence of some galactozymase formation before all the glucose had disappeared.   This is indicated by the fact that relatively vigorous fermentation is observed after 1,000 c. mm. of CO_2 had been evolved. If no galactozymase were present, the slope of the curve would have decreased sharply before this amount accumulated.   A situation of this nature is exhibited by curve C, in which 0.8 mg. glucose was added with the galactose.

Here we see an almost complete cessation of fermentation when about 80 per cent of the glucose added is accounted for, which corresponds to the usual 20 per cent assimilation. Subsequent to this short period of zero activity, galactozymase makes its appearance. It would appear from this that comparatively little active enzyme is formed during the actual fermentation of the glucose. Rapid enzyme formation occurs, after all the glucose has disappeared, with the aid of substances build up during the period of active metabolism.

Not all strains respond equally well to this addition of glucose. Included in Fig. 2 is one case (curve D) in which a $Q_{CO_2}^{N_2}$ of only 6 is reached 6 hours after the addition of 1 mg. of glucose. This is to be compared with 32 for a more responsive strain. This value of 6 is still considerably and significantly higher than that of a control, which attained a $Q_{CO_2}^{N_2}$ of 1 in a comparable period. No strain that we have examined fails to improve its anaerobic adaptability significantly on the addition of adequate amounts of glucose.

In connection with these findings it should be noted that Schultz, Atkin, and Frey (8) state that they observed no stimulating effect of added glucose on the adaptation to galactose under the conditions of their experiment. In general, the experimental set-up of these authors, being on a macro scale, is not comparable to that employed in the present investigation. No details are provided of the conditions under which the effect of glucose was tested, and it is therefore impossible to arrive at a decision on the reason for the difference in results.

Fig. 3 records the results of similar experiments with maltose adaptation under anaerobic conditions. Here the addition of as little as 50γ of glucose results in a significant increase in adaptation rate over the control without any glucose. Amounts equal to or smaller than 25γ were not significantly effective under the experimental conditions described here.

Close inspection of all curves in Figs. 2 and 3 reveals that a decrease in slope occurs on exhaustion of the glucose in the medium. This is made more apparent in Fig. 4, in which are plotted the rates, as $Q_{CO_2}^{N_2}$, attained during the course of the adaptation. The initial steady rate of CO_2 evolution is due to the fermentation of the added glucose. As soon as the CO_2 level is reached which corresponds to the amount of glucose added, a fall in rate occurs, which is then followed by the completion of the adaptive process. It is apparent in the adaptations to both maltose and galactose that, although some adaptive enzyme may be formed while glucose is present, most of it makes its appearance after the glucose is completely consumed.

The question arises whether the presence of the adaptive substrate is necessary during the fermentation of the added glucose in order to obtain the stimulation of adaptation. This was answered by experiments in which the glucose was initially added from one side-arm, while the adaptive substrate was added from another side-arm after all the glucose was fermented (as determined by
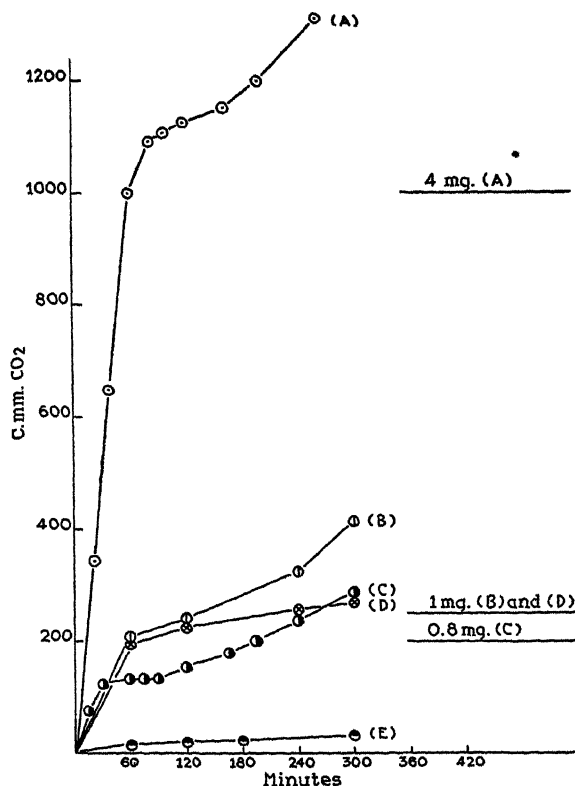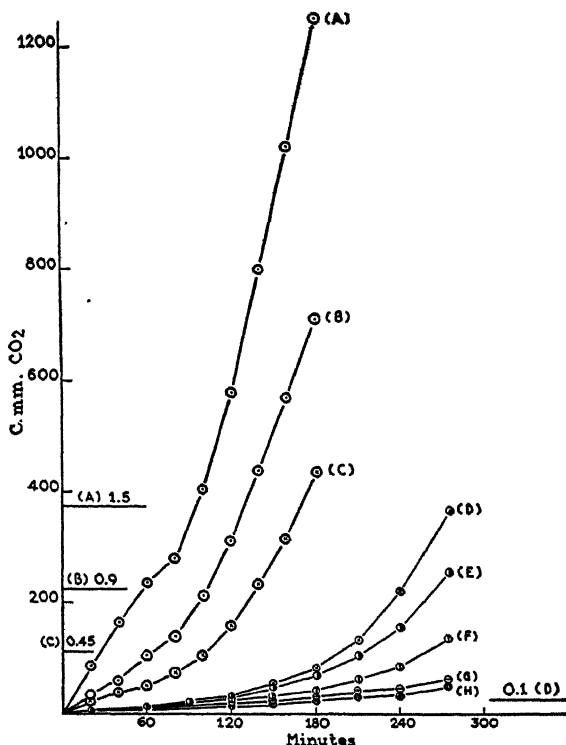
FIG. 3. The facilitation of anaerobic maltose adaptation by small amounts of glucose. (A) 1.3 mg. of added glucose; (B) 0.9 mg. glucose; (C) 0.45 mg. glucose; (D) 0.1 mg. glucose; (E) 0.05 mg. glucose; (F) 0.025 mg. glucose; (G) 0.01 mg. glucose; (H) no added glucose. Horizontal lines indicate theoretical amount of CO_2 corresponding to amount of glucose added.
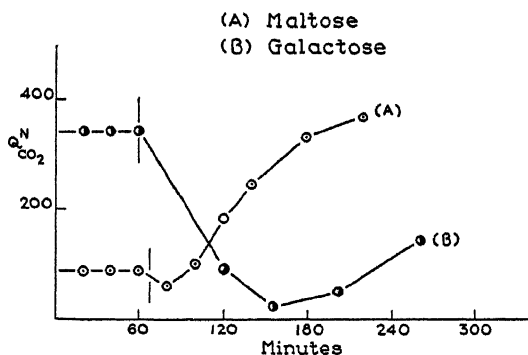
FIG. 4. Rate of fermentation during anaerobic galactose and maltose adaptation in the presence of added glucose, illustrating the drop in rate which intervenes between the exhaustion of glucose and the development of the adaptive fermentation. Vertical lines indicate time at which all the added glucose is accounted for.

the cessation of CO_2 evolution). Fig. 5 gives the results of such an experiment in maltose adaptation in which 1.5 mg. of glucose was used. The control experiment, in which the glucose was added simultaneously with the maltose, shows the usual acceleration of the onset of maltose fermentation. In the case where the maltose was added immediately after the complete fermentation
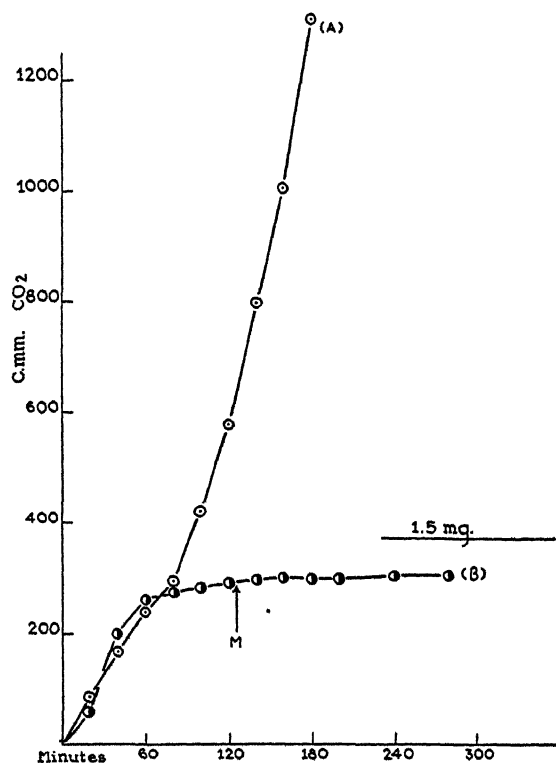
FIG. 5. Dependence of glucose activation of anaerobic maltose adaptation upon the presence of maltose during the glucose utilization. (A) Maltose present from zero time; (B) maltose added after 120 minutes. 1.5 mg. glucose added at zero time in both cases. Horizontal line indicates theoretical amount of CO_2 corresponding to amount of glucose added.

of the added glucose, no such acceleration is observed. Fig. 6 records the results of a similar experiment in adaptation to galactose fermentation, in which 1 mg. of glucose was used. Here again, the mere fact that the cells had fermented the glucose did not aid their anaerobic adaptation to galactose if this fermentation occurred in the absence of the adapting substrate.

All the experiments described so far on the effect of exogenous substrate on anaerobic adaptation have employed glucose. In so far as supplying the energy for the adaptive processes is concerned, presumably any fermentable

'hexose would be suitable, so long as it did not specifically interfere with the fermentation of the adaptive substrate. The data of Leibowitz and Hestrin (15) support this supposition for yeast adapting to maltose. These authors found that any of the fermentable zymohexoses, glucose, fructose, or mannose, was capable of stimulating the adaptation of maltose fermentation.
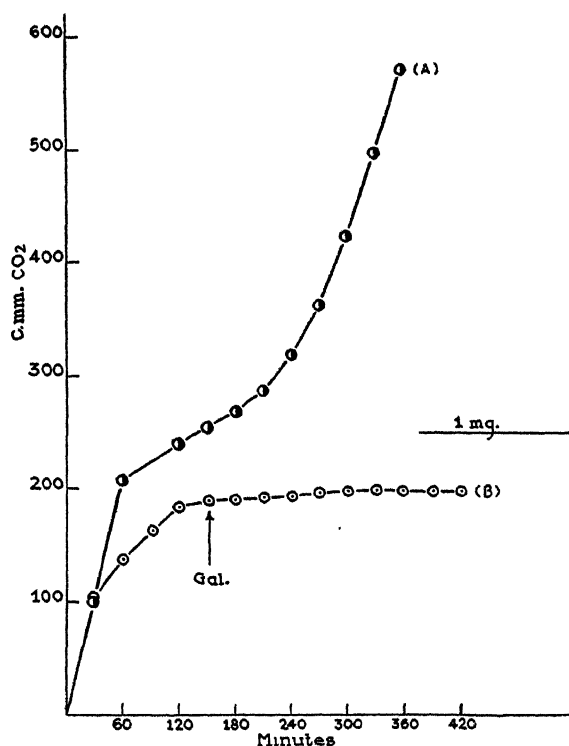
Fig. 6. Dependence of glucose activation of anaerobic galactose adaptation upon the presence of maltose during the glucose utilization. (A) Galactose present from zero time; (B) galactose added after 150 minutes. 1 mg. glucose added at zero time in both cases. Horizontal line indicates theoretical amount of CO_2 corresponding to amount of glucose added.

They further found that galactose, which was not fermented by their yeast preparation, did not aid in the adaptation. From these results it might be deduced that fermentability is the crucial property in determining the suitability as a stimulant of adaptation. Unfortunately, an unequivocal interpretation of the significance of these results cannot be arrived at since the experiments were carried out under aerobic conditions. What was being studied was the combined results of respiratory and glycolytic utilization of the glucose.

The conclusions derivable from these experiments are clearly of great importance to our understanding of the actual nature of the adaptive process. It seemed therefore highly desirable to repeat these experiments under anaerobic conditions and to extend them to the case of adaptation to galactose. In experiments on this question, cultures were employed which possessed no measurable capacity to adapt anaerobically in the time allowed, either to galactose or maltose, in the absence of added fermentable substrate. Table IV records observed $Q_{CO_2}^{N_2}$ at various intervals under the different experimental conditions. In all the instances recorded, 1 mg. of the "exogenous substrate" being tested was added. At 60 minutes, even in the most rapid fermentation, some

TABLE IV

The Effect of Various Substrates on Anaerobic Adaptation to Galactose and Maltose

1 mg. of each substrate was added in every instance. Strain K was employed.

Adaptation to	Exogenous substrate	$Q_{CO_2}^{N_2}$ after:				
		60 min.	120 min.	180 min.	240 min.	300 min.
Maltose	Glucose	100	196	330	374	—
	Fructose	92	145	285	340	—
	Mannose	78	112	240	320	—
	Galactose	1.2	1.1	1.3	1.0	—
	None	1.0	0.9	1.6	1.2	—
Galactose	Glucose	103	13	18	26	37
	Fructose	94	12	22	29	41
	Mannose	80	8	14	25	39
	Maltose	0.9	1.2	1.3	1.2	0.9
	None	1.1	1.0	1.2	1.2	1.0

of this substrate still remains. Consequently, the fermentation rates recorded in the first column (60 minutes) are attributable to the fermentation of the added substrates. Within 80 minutes, however, these are completely consumed in all cases. All subsequent $Q_{CO_2}^{N_2}$ values are therefore due to fermentation of the adaptive substrate. Our results with respect to maltose adaptation are in complete agreement with those reported by Leibowitz and Hestrin (15). Although quantitative differences exist between the effectiveness of the three fermentable zymohexoses, they are all able to stimulate the onset of adaptation. Again, galactose, which was not fermentable by this culture, did not help in the anaerobic adaptation.

As can be seen, essentially the same pattern is obtained in the case of anaerobic adaptation to galactose fermentation. The addition of the three fermentable hexoses resulted in the appearance of galactose-fermenting

capacity. On the other hand, the addition of an equivalent amount of maltose, which was not fermentable by this culture, had no effect.

These experiments suggested the possibility that conditions could be arranged in which galactose could stimulate the anaerobic adaptation to maltose and in which maltose could aid in the anaerobic adaptation to galactose. Since fermentability seems to be the only criterion, it would be predicted that galactose would stimulate a galactose-adapted culture to adapt anaerobically to maltose; and, conversely, maltose would do the same for a maltose-adapted culture adapting to galactose.

TABLE V

The Effect of Maltose and Galactose on Anaerobic Adaptation in Cultures Adapted to One of These Sugars

1.5 mg. of each exogenous substrate were used. The $Q_{CO_2}^{N_2}$ values correspond to those attained 20 minutes after all the added exogenous substrate was accounted for on the basis of the CO_2 evolved.

Adaptation to	Culture	Exogenous substrate	Experiment No.	$Q_{CO_2}^{N_2}$
Galactose	Maltose-adapted	Maltose	1	49
			2	53
		None	1	15
			2	17
Maltose	Galactose-adapted	Galactose	1	84
			2	82
		None	1	9
			2	8

Relatively young (36 hour) cultures were used in these experiments to insure well adapted cells. These experiments employed the same strain used in the experiments recorded in Table IV. Instead of being grown in glucose, however, cultures were grown in maltose and galactose. To minimize loss of the adaptive enzyme during the preparation of the suspensions, care was taken to wash with chilled buffer, and the centrifugation was carried out in the cold. The results are recorded in Table V. In both instances significant stimulation of the adaptive process was attained. The stimulating effects observed here are not as great as with glucose, mannose, or fructose. This is probably due to the fact that the rate of utilization of the added substrate by the adaptive systems is lower than the rate of consumption of the zymohexoses. The data leave little doubt, however, that maltose can accelerate anaerobic adaptation to galactose in maltose-adapted cells, and that galactose can stimulate adaptation to maltose in galactose-adapted cells.

DISCUSSION

The present investigation was undertaken in order to correlate the process of enzymatic adaptation with the energy-yielding metabolic processes of the cell. The implicit assumption is that adaptation requires the formation of some missing components of the enzyme system required to ferment the adaptive substrate. In the case of adaptation to galactose, recent experiments (6) show that the modification involves the apoenzymatic or protein moiety of the enzyme system. Whatever the details of the adaptive process, it is evident that in the yeast cell both the anaerobic and the aerobic oxidative pathways can serve as a source of energy.

(a) The "Activation" Theory of Adaptation

It should be exphasized that our interpretation of the rôle of oxygen in stimulating aerobic adaptation and of glucose in doing the same for anaerobic adaptation is not the usual one offered in the literature. Previous investigators (7, 8) have noted that the presence of oxygen results in a more rapid onset of galactose fermentation by baking yeasts. They attribute this effect to an "activation" of the galactozymase system. The mechanism presumably invoked is the oxidation of some component necessary for the functioning of the galactose-fermenting system.

In the case of adaptation to maltose, positive effects have been seen (8, 15) with oxygen on aerobic adaptation and with fermentable substrates on anaerobic adaptation. Here again, as in the case of galactose adaptation, the explanations are in terms of "activation" of some necessary component.

Such explanations must perforce leave the mechanism of activation vague both as to the nature of the component activated and the precise rôle of the activators. What is, however, more pertinent to the concepts of the present paper is the assumption implicit in all such interpretations of adaptation that the complete adaptive enzyme system exists preformed in the cell but in an inactive state. Adaptation from this viewpoint consists not in the formation or synthesis of some new component but in an activation analogous perhaps to the conversion of trypsinogen into trypsin. It therefore is of interest to examine such hypotheses and the evidence for them.

One immediate difficulty encountered by the activation hypothesis is the necessity for explaining why such diverse agents as oxygen, glucose, galactose, etc., all can activate the dormant enzyme system. The data presented here make the situation even more complicated, since it becomes necessary to explain how the same set of activators can be effective for two entirely different enzyme systems; e.g., galactozymase and maltozymase. Leibowitz and Hestrin (15, 16), in an attempt to get around this difficulty with respect to maltose adaptation, suggest that oxygen and a fermentable substrate would both cause a decrease in inorganic phosphate content, and that this might in some manner lead to the activation of the maltolytic system (28).

It is clear from Fig. 1 and Table III that this simple interpretation of the effect of oxygen is not tenable with respect to either aerobic or anaerobic adaptation. If this were the case, simple exposure to oxygen should lead to the onset of adaptation. We see on the contrary that such pretreatment quickly leads to decreased anaerobic adaptability, and ultimately to an increased lag in aerobic adaptation. Again (Figs. 5 and 6), as concerns the effect of fermentable substrate on anaerobic adaptation, mere exposure to the latter is not sufficient. The important point demonstrated by these experiments is that none of these "activators" is effective, if the cells are exposed to them in the absence of the adaptive substrate. If the mechanism whereby these activators evoked activity were through such a generalized non-specific effect as depression of the inorganic P of the cell, it should not make much difference whether the adaptive substrate were present during their action or immediately after they had accomplished the non-specific effect. The same reasoning can be employed against the suitability of the hypothesis which assumes activation through oxidation of some preexisting but critically necessary component which is inactive in the reduced state. It is difficult to see why the presence of the adaptive substrate should be necessary during the oxidation of a substance which already exists in the cell.

These experiments also make completely untenable a suggestion by Sevag (27). He supposes that the shortening of the induction period in galactose fermentation may be due to the oxidation of the polysaccharide reserves, which might constitute a barrier against galactose. Once these are removed, presumably the galactose could "get at the key enzymes." Again, such an explanation would imply that adaptation would occur independently of the presence or absence of the adaptive substrate during the oxidation. Indeed, it would be expected here also that previous dissimilation should curtail or eliminate the time usually required for adaptation.

The fact that pretreatment with glucose leads to acceleration of galactose and maltose adaptation would seem at first glance to contradict the statement that the adaptive substrate must be present during the metabolism of the activating substrate if stimulation is to occur. However, in such instances, it is the utilization of the polysaccharide formed during the pretreatment period which acts as the accelerator when the adaptive substrate is added. This is supported by the fact that such pretreatments are only effective in stimulating adaptation aerobically; i.e., under conditions permitting the utilization of the carbohydrate reserves. As has been shown (Figs. 5 and 6), they have no effect on subsequent anaerobic adaptation, under which condition the polysaccharide reserves are not effectively utilizable.

There are several other types of experiments which it is pertinent to examine in any attempt to develop an adequate picture of the mechanism of adaptation. It has been demonstrated by several investigators (12, 5) that, if one takes a

culture fully adapted to some substrate (*e.g.*, galactose) and adds glucose, the adaptive enzyme activity begins to fall. Again, if we take a culture fully adapted to galactose, and expose it to oxygen in the absence of the adaptive substrate, the adaptive enzyme activity begins to disappear (5). The same culture will lose relatively little of its enzyme activity if kept under anaerobic conditions. The interesting thing here is that the same agents (oxygen and glucose) which stimulate the appearance of the adaptive enzyme system also accelerate its disappearance. Such diametrically opposed effects of the same agents could be explained only with great difficulty on the basis that they function by activating some preexisting component. In this connection it should be recalled (Fig. 4) that rapid formation of adaptive enzyme stimulated by glucose only occurs *after* the latter has been exhausted from the medium. This again is difficult to reconcile with any simple activating function of the glucose.

Experiments with cell-free extracts are also difficult to explain from the point of view of activation of some preformed element. It has long been known that cell-free extracts may be prepared from galactose-adapted cultures which will actively ferment galactose. Similar preparations from unadapted cultures, while active against glucose, will not ferment galactose. But not one of the "activators" which have been found to stimulate galactose adaptation in the intact cell is effective in evoking such activity from unadapted extracts. This result is not easily reconciled with any "activation" hypothesis, since we know from the experiments that all the essential components of the galacto-zymase system can exist and function in such cell-free extracts.

(b) The Relation of Preformed Enzymes to Adaptation

There are certain experimental results which appear at first to be at variance with those reported on galactose, and which would indicate that enzyme systems can exist *in vivo* in an inactive form and become activated as a result of certain relatively non-specific procedures. It has been possible to extract cellobiase from yeast which failed to exhibit any capacity to ferment cellobiose (17). Invertase has also been demonstrated in extracts derived from *Schizosaccharomyces octosporus*, which is not able to ferment sucrose (18). Similarly, trehalase was obtained from yeast which *in vivo* failed to act on trehalose (19), and hydrolases of melibionic acid and maltose carbonic acid from yeast which could not act on these substrates (20). We might also note that lactase was observed in *Escherichia coli mutabile* variants which were non-fermenters of lactose (21). Finally, yeasts which cannot ferment maltose yield on extraction or drying preparations which possess maltase activity as measured either with maltose or α-methyl glucoside (15).

One might be tempted to take these instances of undoubted activation of preexisting enzyme systems as models of adaptive enzyme formation. There

are, however, certain experimental facts which make it doubtful whether a direct application of these findings to the phenomenon of enzymatic adaptation is possible.

In the first place, it should be noted that all of these instances deal with disaccharide fermentation. The work of Doudoroff and his colleagues (22) has demonstrated that the disaccharide sucrose may be split into its component hexoses in two ways. One is the well known hydrolytic cleavage effected by the enzyme sucrose. The other mechanism involves a sucrose phosphorylase by means of which the sucrose is dissociated into glucose-1-phosphate and fructose.

Furthermore, it was observed (23) that the sucrose phosphorylase is purely adaptive, and can be observed only in preparations derived from cells grown in the presence of sucrose. The hydrolytic enzyme, on the other hand, was a constitutive one, and could be obtained from glucose-grown cells.

Thus, when enzymatic activity on some substrate is observed with unadapted dried cells or extracts as well as with intact cells incubated with the substrate, one may not infer that the enzymes involved are identical. It may be, as it is in the case of sucrose cleavage, that the adaptive enzyme formed by the cells in response to substrate is entirely different from the one which mediates the activity evoked by such procedures as drying and extraction. It is therefore not legitimate to conclude, as do Deere et al. (21), that the same enzyme, lactase, is present in both the fermenting and the non-fermenting variants of E. coli, and that the sole difference between them is that in the fermenting variant the lactase is active, whereas in the negative strain this enzyme is inactive. It is necessary to exclude the possibility that the mutation to lactose fermentation might involve the formation of some other lactose-splitting enzyme. This possibility is pointedly emphasized by the finding by Monod and Lwoff (24) that the mutation per se is not sufficient to result in the appearance of enzyme activity. Previous incubation with the substrate is essential if the enzyme is to be observed. In other words, mutation results in the capacity to form an adaptive enzyme enabling the cell to ferment lactose, and is not simply a question of an activation by the mutation of a preexisting enzyme which is present in an inactive form. We have here in the lactose-positive strain a case strikingly analogous to the sucrose one, in which an adaptive system using the same substrate may be induced in the same cell.

The investigations of Leibowitz and Hestrin (15) on maltose fermentation in yeast indicate strongly that this situation obtains also in the case of adaptation to maltose. These authors found that the enzyme system which appears on contact of the yeast cells with maltose does not possess the properties expected of maltase, the hydrolytic maltose-splitting enzyme which can be detected in dried unadapted yeast. Maltose-adapted cells ferment maltose at a maximum rate at pH 5.0, a hydrogen ion concentration which would

drastically inhibit maltase. On the other hand, no fermenting capacity was observed at pH 7.0, where maltase is maximally active. Furthermore, maltose-adapted cells could not ferment α-methyl glucoside, a specific substrate for maltase under the conditions of the experiment. It seems necessary to conclude here again that the adaptive enzyme formed during incubation with substrate is different from the constitutive hydrolytic enzyme demonstrable in the extract.

Finally, the results of experiments on galactozymase in cell-free extracts of adapted and unadapted cells (6) make it extremely unlikely that any pre-formed enzyme occurs in this important case.

In view of the foregoing considerations, all these instances of activation of preexisting enzymes by procedures which destroy or disturb cell structure may be quite misleading, and it would be hazardous to accept them as models of the adaptive process. They certainly cannot be used as evidence against the hypothesis that enzymatic adaptation involves the formation of a new enzyme component.

(c) The Rôle of the Specific Substrate in Enzymatic Adaptation

It seems evident that a crucial aspect of the adaptive process is the rôle of the adaptive substrate. Inherent in this same aspect of the problem is the specificity of the adaptation with respect to the adaptive substrate. The fact that the latter must be present during the active metabolism of either the endogenous or exogenous substrates is convincing evidence of its critically directive rôle. Certainly one of the most serious criticisms that can be leveled at the activation hypotheses is their failure to provide a mechanism for this feature of the adaptive process which is its most unique and outstanding characteristic.

The critical rôle of maltose in maltose adaptation was clearly recognized by Leibowitz and Hestrin (15, 16, 29). They point out that, while treatment with glucose shortens the induction period prior to maltose adaptation, it does not abolish it. They therefore suggest that some catalyst derived from the maltose must be formed. The nature of this catalyst is, however, not further detailed. Some hint as to its nature may perhaps be gained from some of their results. They find that α-methyl glucoside inhibits the onset of the maltose fermentation, although it has little effect on the fermentation once it is started. Further, β-methyl glucoside has no effect on the maltose adaptation. Pretreatment with glucose does not abolish the inhibitory effect of the α-methyl glucoside. High concentrations of maltose can partially overcome the α-methyl glucoside inhibition.

It would seem from these results that, in the early stages of the adaptation, α-methyl glucoside can compete successfully with the maltose for some active group. Combination with this active group by maltose is apparently essential for the progress of the adaptation. The α-methyl glucoside thus may function

as an inactive analogue of maltose and by tying up the available centers prevent their combination with maltose. The fact that β-methyl glucoside cannot so function is evidence that the active groups being competed for are specific for α-glucoside linkages. The catalyst whose formation is postulated by Leibowitz and Hestrin may therefore well be an enzyme which can unite with compounds containing the α-glucosidic link.

The available data on enzymatic adaptation can be consistently explained on the hypothesis that enzyme formation is involved. The fact that the presence of substrate is necessary can be understood on the basis that combination between the substrate and the enzyme is essential for the synthesis of more enzyme. The fact that the kinetics of the appearance of enzyme activity is autocatalytic (3, 30, 31) has been taken to signify the existence of a self-duplicating enzyme-forming system (3, 4). In any case, the simple mass action law proposed by Yudkin (25) seems inadequate to explain the facts. This hypothesis has been criticized on other grounds by Monod (32), who suggests that adaptation involves the combination of substrate with relatively non-specific enzyme precursor (26).

It has been proposed (5) that the autocatalytic system observed is a cyto-plasmic component consisting of a plasmagene-enzyme complex which is stablized by substrate. The data presented in the present paper on the relation between adaptation and the metabolism of exogenous and endogenous substrates are consistent with the general view that enzyme adaptation involves protein modification requiring energy-yielding metabolic reactions.

SUMMARY

The source of energy for enzymatic adaptation has been investigated. Aerobically, it is found that the endogenous carbohydrate reserves may be used as such a source. In cells depleted of their reserves, the adaptive substrate itself can be oxidized even while it cannot be fermented, and so can serve as a source of energy for the adaptation to a fermentative mode of utilization.

Anaerobically, adaptation may occur at the expense of stored energy-rich compounds, while the reserves and the adaptive substrate are now useless as fuel. Such compounds appear to be more plentiful in young than in old cells. The addition of any fermentable substrate, such as glucose, leads to rapid anaerobic adaptation. Experiments in which maltose-adapted cells are adapted anaerobically to galactose with the aid of a little added maltose, and conversely, show that fermentability is the criterion of usefulness for an exogenous substrate in aiding the adaptive process.

None of the endogenous and exogenous energy sources which have been investigated will facilitate adaptation unless the adaptive substrate is present while they are being consumed.

The significance of these findings and the adequacy of "activation" hypotheses to explain enzymatic adaptation has been discussed.

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THE PREADAPTIVE OXIDATION OF GALACTOSE BY YEAST*

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INTRODUCTION

It has been demonstrated (1) that the adaptation of yeast to ferment galactose involves a modification of the apoenzymatic or protein portion of the enzyme system. A transformation of this kind would presumably require an energy supply.

This conclusion is supported by a number of facts. Adaptation of yeast to ferment a substrate such as galactose is relatively rapid under aerobic conditions (2, 3). Under anaerobic conditions, adaptation by cells from a 48 hour culture is either enormously slower or does not occur at all. This can be correlated with the finding (4-6) that the intact yeast cell is unable to use its endogenous carbohydrate reserves anaerobically, whereas the latter are quite readily oxidized aerobically. The correlation was established more directly by experiments in which the carbohydrate stores of the cells were exhausted to various degrees by varying times of aeration in the absence of exogenous substrate. During this process, the rate of endogenous respiration diminishes. The time of adaptation increases steadily with increasing exhaustion of the endogenous reserves, and rises quite sharply as the endogenous approaches zero.

Finally, it has been shown (7) that, if anaerobically available energy be supplied in the form of a small amount of fermentable substrate, anaerobic adaptation is effected under conditions which would otherwise not support the adaptive process.

As has been pointed out previously, one of the paradoxes of the aerobic adaptation lies in the fact that it can occur after virtually complete exhaustion of the carbohydrate reserves. While it is possible that stored fat and protein could under such condition be utilized by the cell as a source of energy for synthetic activity, observation of oxygen consumption and CO_2 production gives no evidence that such is the case. A resolution of this paradox was offered by the finding (8) that oxidation of the adaptive substrate could occur before the appearance of any of the enzymes necessary to its fermentation. This

capacity of unadapted cells to oxidize the substrate suggested the possibility that this preadaptive oxidation was the source of energy for the adaptive process.

It is the purpose of the present paper to provide further evidence for the existence of this preadaptive oxidation as well as to explore some of the details of the reaction.

Methods and Materials

The strains employed and the methods of handling the cultures are similar to those detailed in the previous paper. The same can be said concerning the manometric measurements and methods of preparing standard suspensions. The galactose employed in the present investigations was purified by the method previously described. The purity of the resulting preparation was tested by an analysis for the presence of contaminating fermentable substances by the method of Winzler (9). Less than 0.05 per cent of such material was present in the galactose used in the experiments reported here. Reducing sugar was determined by the method of Folin and Malmros (10). When galactose was being determined, galactose standards were employed.

In the experiments in which carbon balances were sought, the following procedure was used. Subsequent to deproteinization the several groups of components were separated by a number of steps according to the following plan.

1. A distillation of a measured quantity was made at an acid pH (acid to Congo red). The condenser (water-cooled) was equipped with an adapter which dipped below the surface of a few milliliters of ice cold distilled water in the receiver, the latter being surrounded by an ice bath. The solution was distilled down to one-third of its original volume. This distillation separates part of the volatile organic acids and all the ethyl alcohol and acetaldehyde from non-volatile substances such as galactose.

2. The distillate was made alkaline (a small amount of solid powdered phenolphthalein was used as an indicator) with NaOH, and redistilled in the same fashion as above. This separates the alcohol and acetaldehyde from the volatile acids, whose Na salts are non-volatile. The distillate was made up to a definite volume, to be further analyzed for alcohol.

3. The residues of the first and second distillations were again made acid to Congo red, and once more distilled. The combined residue, whose volume might be 25 to 50 ml., was first distilled down to a volume of 15 ml., and then subjected to a steam distillation until ten volumes (150 ml.) had come over. The combined distillate contains the volatile acid fraction.

4. The residue from the third distillation was subjected to a continuous ether extraction overnight (12 to 18 hours). The ether extract was then evaporated on the steam bath, and the residue taken up in distilled water and brought to a definite volume. This fraction contains non-volatile but ether-soluble organic compounds, particularly the dicarboxylic and lactic acids. The ether-insoluble residue of the extraction contains chiefly carbohydrates and inorganic salts.

The point of the procedure just outlined is that, at the cost of some extra steps, it permits the separation of the volatile compounds into the two classes of alcohol plus acetaldehyde and volatile acid without incurring the risk of caramelization of residual carbohydrate which would occur during heating at an alkaline pH.   .

The steam distillation (step 3) will probably carry over about a third of any pyruvic

acid which may be present. If the amount of pyruvic acid is appreciable, a second steam distillation is required to effect a reasonably good separation of pyruvic from acetic and the other volatile acids. Since qualitative tests for pyruvic acid on aliquots of the original solution and the various fractions by the Na nitroprusside method (11) were completely negative, this additional step was not required.

The alcohol fraction required one further step before analysis. This consisted in oxidizing the contents of a 50 ml. aliquot with acid dichromate by heating for 10 minutes in a boiling water bath. This was followed by a ten volume steam distillation conducted as in step 3 above. The oxidation converts the alcohol and aldehydes into the corresponding acids, which are found in the steam distillate.

Each of the fractions is now determined by titration of the acids present with standard alkali (about 0.02 N NaOH). In the case of the alcohol fraction, the equivalents of acid obtained by titration are divided by 0.984 (if only ethyl alcohol and acetaldehyde are present) to obtain the equivalents of alcohol (12).

The individual acids in the non-volatile fraction were not all determined. Lactic acid was investigated by the colorimetric methods of Barker and Summerson (13) and found to be absent, while pyruvic acid was eliminated as mentioned earlier. This leaves no common monocarboxylic acids to be considered.

Preadaptive Galactose Oxidation

A typical adaptation curve obtained with strain 812 is presented in Fig. 1. The results found with other strains do not differ in any but the minor quantitative aspects to be expected from differences in the induction periods and the levels of the endogenous respiration. As indicated by the arrow, the galactose was added at the very beginning of the experiment. The addition produced no significant change from the endogenous respiration. The Q_{O_2} in the initial portion of the curve of Fig. 1 is close to the value of 28.0 for the endogenous Q_{O_2} obtained in a parallel run with a control set to which galactose was not added. The absence of immediate effects on the addition of galactose is also observed in Fig. 2 where the addition occurred 50 minutes later. Nevertheless, it is evident, particularly from Fig. 2, that the galactose is affecting the physiological characteristics of the cell even before the enzymatic apparatus necessary for its fermentative utilization makes its appearance. Normally the endogenous respiration would have begun to show a sharp decrease at 120 minutes. At the end of 150 minutes the Q_{O_2} of the endogenous control run at the same time had decreased to 40 per cent of its initial value. No such change is observed in Fig. 2 during the same period.

The nature of the phenomenon involved here is more clearly seen in Fig. 3, where the galactose was added after the cells had entered the zero-rate portion of the endogenous curve. Immediately following the addition, there is a considerable burst of O_2 consumption and CO_2 evolution. Subsequently the respiration rate assumes the value characteristic of the linear portion of the endogenous curve. This is maintained until either adaptation occurs or the substrate is exhausted.

Table I records a more extensive survey of this phenomenon covering the entire endogenous curves of two strains in intervals of 30 minutes. It is clear from the results obtained that the presence or absence of an effect of the addition of galactose on the respiratory rate depends on the level of the endogenous respiration at the time of the addition. If the latter is maximal, no further
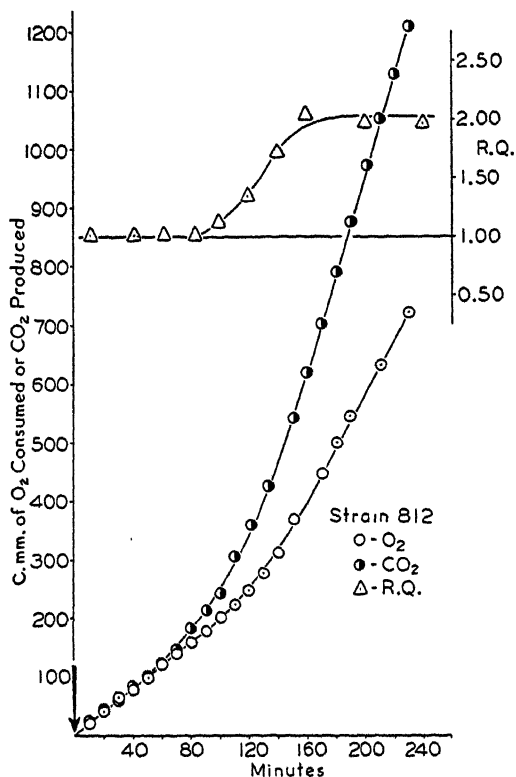
FIG. 1. A detailed examination of the course of aerobic adaptation. The arrow indicates the point of addition of the galactose. The upper curve gives the R. Q. calculated from the data of the two lower curves.

increase is observed. If it is low, however, the addition of galactose will raise it to the levels attained during the linear portion. It will further be observed that these effects of galactose on the aerobic metabolism are not reflected in any changed capacity to handle the sugar anaerobically. During these periods when active aerobic oxidation was observed with galactose as the sole substrate, the $Q_{CO_2}^{N_2}$ values in the presence of galactose remained uniformly negligible. It will further be noted that, in all the cases examined, the R.Q. did not differ

significantly from unity, either in the high rate period immediately following the addition of the galactose or in the subsequent one where Q_{O_2} and Q_{CO_2} values characteristic of the constant-rate portion of the endogenous curves were assumed.

FIG. 2. Course of aerobic adaptation. The arrow indicates point at which the addition of galactose was made. Illustrating the absence of effect of galactose on Q_{O_2} during the linear phase of endogenous respiration.

Certain of the details in the response to galactose in the preadaptive period were different when other strains were used. Fig. 4 illustrates an experiment of this kind with strain C1, which is a representative of *S. carlsbergensis*. This culture normally has a rather high endogenous respiration, attaining Q_{O_2} values of 55. In the experiment depicted in Fig. 4, a culture of this kind was dissimilated until it reached a Q_{O_2} value of about 5.0. This is the rate observed in the first 60 minutes in Fig. 4. On the addition of galactose at 60 minutes the respiratory rate increases to 28 and subsequently rises higher. Unlike the *cerevisiae* strains described in the previous curves, no sudden burst of oxidation

is observed and the respiratory rate characteristic of the linear portion is not attained until some time after contact with galactose is made. This lowered response of strain C is attributable to the circumstance that this yeast is physiologically less vigorous than the strains of *S. cerevisiae* with which it is compared, and is rather easily injured by the process of dissimilation.
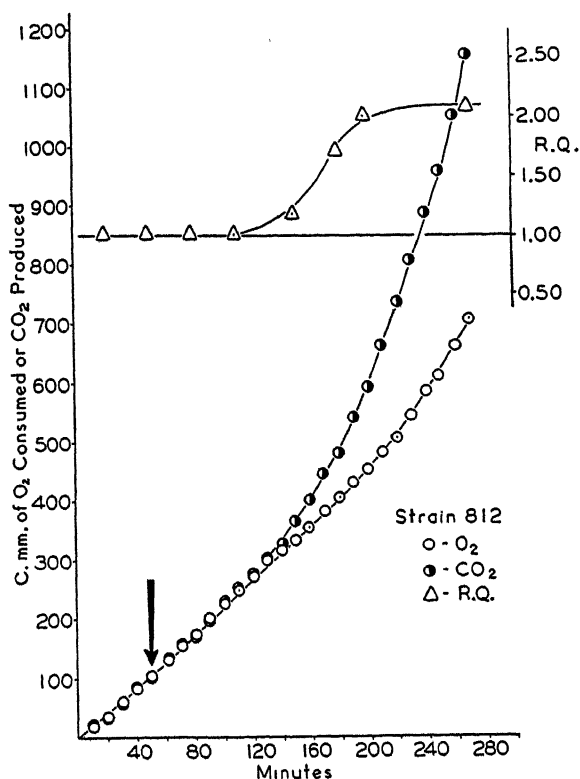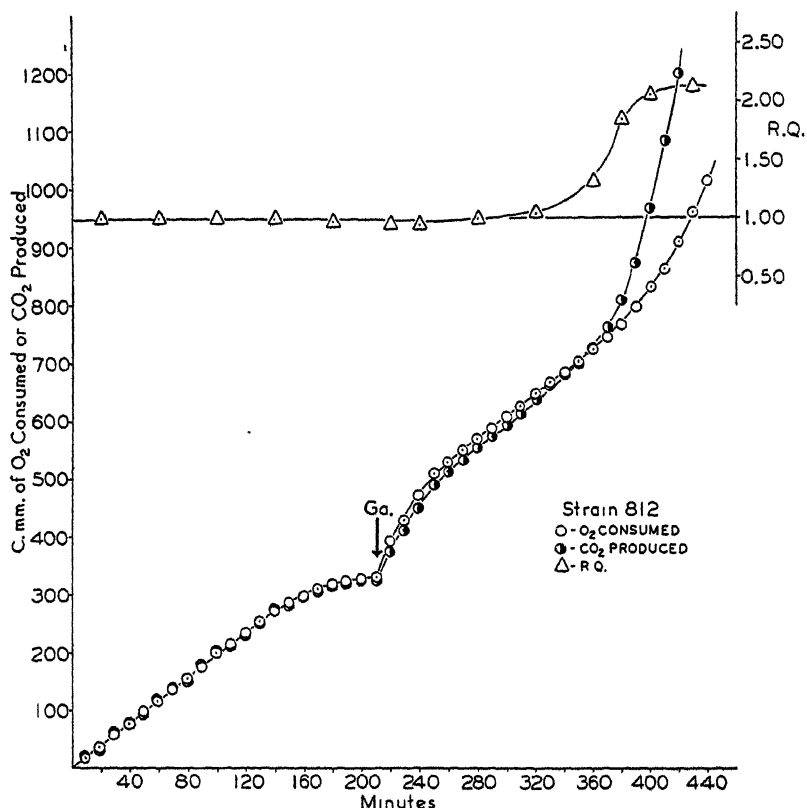

FIG. 3. Course of aerobic adaptation. The arrow indicates point at which the addition of galactose was made. Illustrating the stimulation of Q_{O_2} by galactose after exhaustion of endogenous respiration.

These experiments strongly suggest that in all the strains examined there exists a mechanism for preadaptive utilization of galactose. The unity of the R.Q. and the complete absence of the ability to produce CO_2 anaerobically further points to a purely aerobic metabolism in this period. The fact that the addition of galactose fails to lead to respiratory stimulation during the linear endogenous period would seem to indicate that the preadaptive metabolism of the galactose follows the same pathway as that used in the oxidation of the carbohydrate reserves, and that these kinds of substrates compete for enzyme

TABLE I

Effect of Adding Galactose during Different Periods of the Endogenous Respiration on the Q_{O_2}, $Q_{CO_2}^{O_2}$ and $Q_{CO_2}^{N_2}$ Values of Unadapted Cells

| Strain	Period at end of which addition was made	Before addition			20 min. following addition			40 min. following addition		
		Q_{O_2}	$Q_{CO_2}^{O_2}$	$Q_{CO_2}^{N_2}$	Q_{O_2}	$Q_{CO_2}^{O_2}$	$Q_{CO_2}^{N_2}$	Q_{O_2}	$Q_{CO_2}^{O_2}$	$Q_{CO_2}^{N_2}$
	min.									
812	0–30	27.3	27.3	0.01	27.3	27.3	0.1	27.3	27.3	0.1
	30–60	27.6	27.7	0.1	27.7	27.7	0.1	27.7	27.6	0.1
	60–90	27.0	26.3	0.3	26.5	26.5	0.0	26.5	26.5	0.2
	90–120	24.3	24.0	0.08	26.8	27.1	0.2	27.2	27.0	0.2
	120–150	12.2	12.1	0.1	27.5	27.0	0.2	27.5	27.1	0.1
	150–180	9.1	9.0	0.1	38.0	38.2	0.3	26.8	27.2	0.0
	180–210	6.2	6.2	0.2	56.3	53.3	0.1	29.1	28.3	0.1
	210–240	2.1	1.9	0.2	70.2	65.3	0.2	29.3	29.8	0.1
LK2G12	0–30	31.2	31.3	0.1	31.3	31.3	0.1	31.2	31.3	0.1
	30–60	31.3	31.3	0.3	31.3	31.1	0.3	31.3	31.1	0.3
	60–90	32.4	32.0	0.4	32.4	32.0	0.4	33.0	32.0	0.4
	90–120	31.0	30.8	0.8	33.1	32.0	0.6	33.1	32.0	0.6
	120–150	27.0	27.0	0.1	40.1	39.5	0.2	31.6	32.3	0.2
	150–180	15.4	15.5	0.2	53.2	50.7	0.5	32.4	33.1	0.5
	180–210	9.0	9.1	0.03	78.9	76.3	0.03	30.5	30.8	0.03
	210–240	5.2	5.4	0.01	85.6	84.0	0.05	34.3	32.1	0.01
	240–270	0.8	1.0	0.03	90.1	87.3	0.07	32.6	31.0	0.03
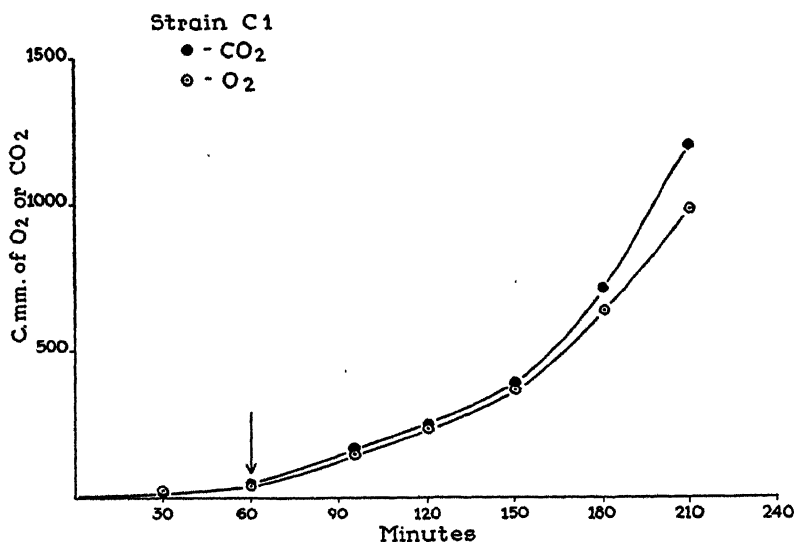
FIG. 4. The effect of adding galactose on the aerobic metabolism of a dissimilated culture of *S. carlsbergensis*.

when both are present together. The fact that the Q_{O_2} can rise above this level lends further support to this interpretation. If such a competitive interaction did exist, a preferential utilization of the carbohydrate reserves would leave the rate unchanged on the addition of galactose during a period when the concentration level of the preferred substrate is non-limiting.
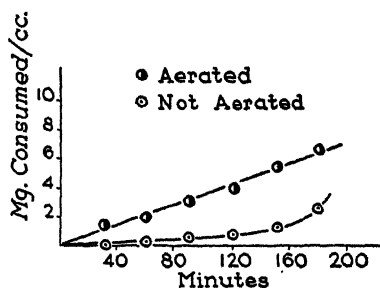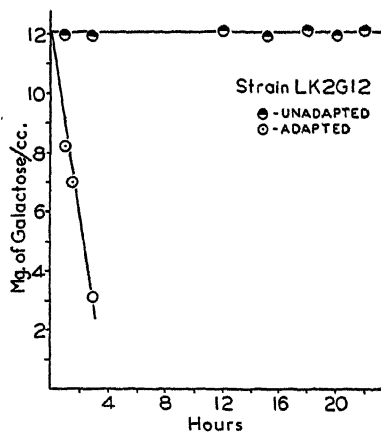

FIG. 5   FIG. 6

FIG. 5. Preadaptive aerobic metabolism of galactose by dissimilated and undissimilated cells. The ordinate represents the amount of galactose consumed by 1 ml. of the suspension, expressed in milligrams. Illustrating the rapid preadaptive consumption of galactose by dissimilated cells.

FIG. 6. The anaerobic assimilation of galactose by adapted and unadapted cultures. The ordinate represents the residual galactose in milligrams, in 1 ml. of the suspension. Gas phase N_2. Illustrating the inability of unadapted cells to consume any galactose.

Relation between Galactose Oxidation and the Endogenous Respiration

The hypothesis of interaction between the oxidation of galactose and of the endogenous reserves was tested by experiments which examined and compared the metabolism of galactose in cultures prepared under different conditions.

In these experiments 2 day cultures were washed as usual and resuspended in $M/15$ KH_2PO_4. The resulting suspension was divided into two portions. One received an amount of galactose sufficient to make a final solution containing 25 mg./cc. and was then placed in a water bath at 30.2°C. and shaken. Samples were withdrawn at intervals for sugar analysis. The other portion of the suspension was allowed to dissimilate for 4 hours while shaking in a water bath. At the end of this period the same amount of galactose which was used previously was now added to this portion, and the carbohydrate content was subsequently followed. The results of such an experiment with one of the strains employed are given in Fig. 5. Two other strains were tested in the same manner and gave similar results.

It is evident from Fig. 5 that previous dissimilation, resulting in the exhaustion of the endogenous reserve, facilitates the preadaptive utilization of the galactose. The departure from linearity observed in the lower curve represents the onset of adaptation and the fermentative utilization of the galactose. These results support the conclusion concerning the relation between this preadaptive utilization of the galactose and the endogenous respiration.

This phenomenon of the preferential utilization of the endogenous reserves can cause the appearance of an "induction" period in the utilization of an externally supplied substrate. Such an "induction" may have all the characteristics of adaptation to the utilization of the exogenous substrate. However, such "adaptations" can easily be distinguished from true adaptations, since they can be made to occur by dissimilation in the absence of the substrate to which the cell is presumably becoming adapted.

TABLE II

Galactose Disappearance under Anaerobic Conditions with Unadapted Cultures

Experiment	Strain	Galactose per cc. at 0 time	Time in contact	Galactose per cc.	Galactose assimilated
		mg.	*hrs.*	*mg.*	*mg.*
1a	Db23B	14.7	6.3	14.8	0.
1b	Db23B	14.7	20.7	14.6	0.1
1c	Db23B	20.0	4.3	20.0	0.
2a	LK2G12	17.5	4.7	17.4	0.1
2b	LK2G12	17.5	4.7	17.3	0.2
3a	812	20.0	6.0	20.0	0.0
3c	812	14.8	23.5	14.7	0.1

It was pointed out above that the unity of the R.Q., as well as the absence of any anaerobic CO_2 production during the preadaptive period, strongly suggested that during this period galactose is metabolized by a purely aerobic mechanism. Further experiments were performed to test this supposition more directly.

Suspensions of 2 day cultures were prepared in the usual manner and dissimilated for 3.5 hours at 30.2°C. They were then distributed to Warburg vessels with known amounts of galactose in the side-arms. After flushing with nitrogen, the galactose was tipped and the vessels were allowed to shake. After various intervals, pairs were removed, the yeast quickly centrifuged down, and the supernatant analyzed. The results are shown in Table II.

A variation of this experiment was also carried out which involved longer incubation periods.

Two day glucose-grown cultures were treated and suspended as above. Samples were then distributed to Thunberg tubes containing known amounts of galactose in their side-arms. The tubes were evacuated and filled with nitrogen twice. After

having been brought to the temperature of the water bath the galactose in the side-arms was tipped into the main compartments of all tubes simultaneously. At intervals, one after the other was removed from the water bath, the yeast quickly centrifuged, and an aliquot of the supernatant taken for analysis.

Fig. 6 gives the results obtained with one of the strains employed. For purposes of comparison, data obtained with an adapted culture of equal density under similar conditions are included. The sole difference in the treatment of the adapted culture is that it did not undergo any prior dissimilation. Such dissimilation could not be employed since the galactozymase system is extremely unstable and tends to disappear rapidly in oxygen in the absence of its substrate.

It is clear, both from Table II and Fig. 6, that no significant metabolism of galactose occurs under anaerobic conditions with unadapted cells. Fig. 6 further illustrates the inability of certain strains to adapt anaerobically even with prolonged incubation with substrate.

The Effect of Inhibitors on the Respiration of Galactose by Unadapted Cells

To check further on the nature of the respiration of the unadapted cell in the presence of galactose, the effects of the addition of cyanide on the Q_{O_2} and $Q_{CO_2}^{N_2}$ were compared.

In these experiments the cells were first dissimilated for 3.5 hours to reduce the contribution of the endogenous respiration to less than 10 per cent. Sufficient galactose was added to yield a final concentration of 4 per cent. By allowing 30 minutes to elapse after the addition of the galactose before the experiment was started, the "burst portion" (see Fig. 3) of the galactose oxidation was avoided. All the data were obtained during the stable linear portion of the curve. The suspending fluid in these experiments for both the experimentals and controls was M/15 phosphate buffer at pH 7.2. The oxygen cup contained appropriate mixtures of KOH and HCN in the center well to avoid net distillation from the main compartment.

The results of these experiments are given in Table III. They indicate that the CO_2 produced is purely respiratory in origin, since the effects on the oxygen consumption and CO_2 production parallel one another.

Lundsgaard (14) demonstrated the specific effect of low concentrations of iodoacetic acid (IAA) on the fermentative process in yeast. He found that 10^{-4} M IAA completely inhibits the fermentation, leaving the respiration unhampered, the R.Q. approaching unity. This offered another method of testing for the existence of fermentative components in the preadaptive metabolism of galactose. Here again, as in the case of the cyanide experiment, the cells were previously dissimilated to insure that the major portion of the respiration being studied was due to the galactose added.

These experiments were performed with cells suspended in $M/15$ KH_2PO_4 containing 4 per cent galactose. Precautions, similar to those taken in the cyanide experiment, were observed here to insure that the observations were being made in the linear portion of the curve. Readings were recorded 50 minutes after the introduction of the IAA from the center well into the main compartment. This delay was made to compensate the lag effect often observed in inhibitions of oxygen consumption due to the addition of IAA.

TABLE III

Effect of KCN on Respiration of Unadapted Cells in 4 Per Cent Galactose

Strain	Control		KCN concentration	Experimental		Inhibition	
	Q_{O_2}	Q_{CO_2}		Q_{O_2}	Q_{CO_2}	Q_{O_2}	Q_{CO_2}
						per cent	*per cent*
LK2G12	31.0	31.00	1×10^{-3} M	1.9	2.3	93.9	92.6
LK2G12	30.5	31.2	1×10^{-2} M	0.3	0.0	96.6	100.0
812	26.3	27.2	1×10^{-3} M	2.4	1.3	90.8	95.2
812	26.8	26.5	1×10^{-2} M	0.01	0.6	100.	97.0

TABLE IV

Effect of IAA on Respiration of Unadapted Cells Metabolizing Galactose

The Q_{O_2} and Q_{CO_2} values reported represent those attained 60 minutes after the introduction of the IAA.

Strain	IAA concentration	Q_{O_2}	Q_{CO_2}	Inhibition	
				Q_{O_2}	Q_{CO_2}
				per cent	*per cent*
812	0	27.1	27.0	—	—
	2×10^{-4} M	27.1	27.0	0	0
	2×10^{-3} M	10.3	10.1	62	61
	2×10^{-2} M	0.0	0.0	100	100
	0	31.5	32.1	—	—
	2×10^{-4} M	31.5	32.1	0	0
	2×10^{-3} M	10.7	9.9	66	69
	2×10^{-2} M	0.0	0.0	100	100

From the results summarized in Table IV, it is evident that no selective inhibition on the carbon dioxide production occurs, as would be the case if the origin of the CO_2 were fermentative. Figs. 7 and 8 give the detailed results obtained with strain LK2G12. For comparative purposes, the curves obtained with adapted cultures of the same strain are also included. These curves demonstrate even more clearly than is possible by a table the striking difference in the behavior towards IAA of cells fermenting galactose as compared with
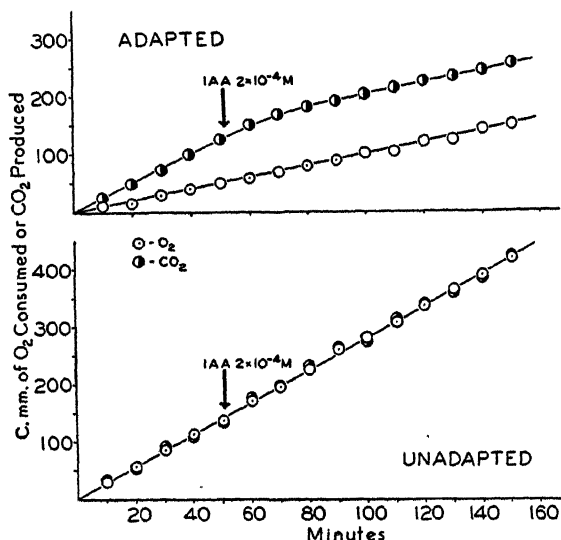
FIG. 7. The effect of IAA on the Q_{O_2} and $Q_{CO_2}^{O_2}$ of adapted and unadapted yeast-metabolizing galactose. The galactose was introduced 40 minutes prior to the first reading. This illustrates the effect of an IAA concentration (2×10^{-4} M) too low to inhibit the purely oxidative component of the metabolism.
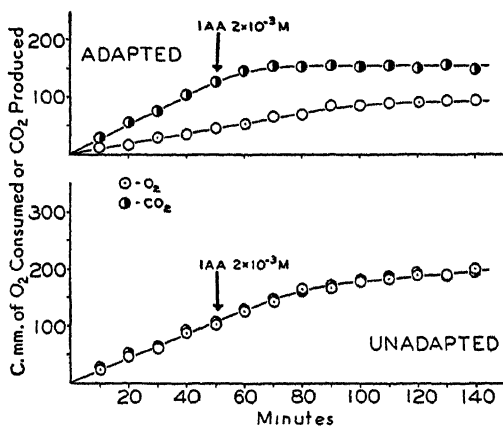
FIG. 8. The effect of IAA on the Q_{O_2} and $Q_{CO_2}^{O_2}$ of adapted and unadapted yeast-metabolizing galactose. The galactose was introduced 40 minutes prior to the first reading. This illustrates the effect of an IAA concentration (2×10^{-3} M) high enough to inhibit the oxidative as well as the fermentative component of the metabolism.

those metabolizing it by means of a purely aerobic system. Where the IAA is sufficiently high in concentration to affect the aerobic process, the oxygen

and CO_2 curves parallel each other, as in the case of the unadapted culture oxidizing galactose in the presence of a 2×10^{-3} M IAA (lower curve, Fig. 8). Such a parallel response is not seen with the adapted culture (upper curve, Fig. 8). In this latter case CO_2 production responds more quickly and more markedly than the oxygen consumption. It will be noted from Fig. 7 that 2×10^{-4} M IAA, which is unable to affect the aerobic utilization of galactose by the unadapted cells, causes a decrease in the aerobic fermentation resulting in a final R.Q. of 1. That this concentration of IAA is able completely to inhibit the fermentative utilization of galactose by adapted cultures was further tested by examining its effect on anaerobic production of CO_2. In all cases a $Q_{CO_2}^{N_2}$ of zero was obtained within 10 minutes following the addition of the IAA.

In general, the response of the galactose respiration is strikingly similar to that found for the endogenous respiration (4, 5).

The Assimilation of Galactose and the Products of Its Utilization by Unadapted Cells

It has been shown in the preceding section that galactose can be oxidized by unadapted cells, and demonstrated directly that galactose disappears from the medium during the preadaptive phase. The question naturally arises whether the oxygen consumed corresponds to the galactose which has disappeared. The usual method for testing the completeness of the oxidation of a substrate involves introducing a known amount and comparing the oxygen consumed with that which would be predicted theoretically from the amount added. In most cases it is possible to determine the point at which all of the added substrate has been consumed by the occurrence of a sharp drop in the respiratory rate to the endogenous level at the point of exhaustion of the exogenous substrate. In the present instance, however, this method is not available, since the respiratory rate of unadapted cells metabolizing galactose does not differ significantly from the endogenous rate. Under these circumstances a very gradual decrease is observed on the exhaustion of the external substrate, and not only is its initiation hard to detect, but it is probable that the fall occurs some time after all of the exogenous substrate is exhausted. In view of this fact it was necessary to make parallel determinations of oxygen consumption and galactose disappearance.

The 2 day cultures used in these experiments were dissimilated for 7 hours to insure negligible contributions from the endogenous respiration. No experiment was run over 3 hours after the introduction of the galactose, in order to avoid overlapping into the adaptive period when fermentative utilization occurs.

The procedure adopted after some preliminary experiments was as follows: Portions of the dissimilated suspensions were set up in Warburg vessels for oxygen consumption and CO_2 production measurements. After the galactose was transferred from the side-arms to the main compartment, the vessels were allowed to shake for various periods of time. During this incubation period the course of the oxygen

consumption and CO_2 production was followed manometrically. When an adequate amount of oxygen had been consumed, the vessels were removed and the contents centrifuged quickly in preparation for an analysis of the supernatant for the remaining galactose. The values thus obtained for two strains are recorded in Table V.

It is evident that a considerable discrepancy exists between the oxygen consumed and that which would be expected on the basis of the amount of galactose which had disappeared. There are several possible ways of interpreting results of this nature. It is conceivable that synthesis of cellular components accompanies the oxidation of the galactose. Such phenomena have been observed by Winzler and Baumberger (15), Winzler (16), and van Niel and Cohen (17) in yeast consuming other substrates. A similar discrepancy between oxygen consumed and substrate metabolized has been noted in other micro-

TABLE V

Oxygen Consumption Corresponding to the Metabolism of Various Amounts of Galactose

The theoretical amount of oxygen required for the complete oxidation of 1 mg. of galactose to CO_2 and H_2O at 30°C., the temperature of the experiment, is 748 c.mm.

Strain	Galactose consumed	O_2 consumed	R.Q.	Theoretical	Recovery
	mg.	*c. mm.*		*c. mm.*	*per cent*
812	2.1	455	1.00	1570	29
	1.6	406	1.10	1120	34
	3.5	838	1.08	2620	32
LK2G12	3.0	695	1.00	2240	31
	2.2	592	1.08	1640	36
	1.8	443	1.03	1340	33

organisms by Barker (18), Clifton (19), Clifton and Logan (20), and Doudoroff (21). These authors have also interpreted their observations in terms of the assimilation of the unaccounted for substrate as cellular material. However, it must be noted that, despite the unity of the R.Q., all or part of the discrepancy might be ascribed to incomplete oxidation of the galactose.

In order to examine this question further an attempt at a complete carbon balance was made, in which gas production, galactose utilization, polysaccharide storage, and the formation of alcohol and organic acids were studied. At the same time a comparison was made between the preadaptive and postadaptive products of galactose utilization. The following experimental procedure was adopted.

Several Erlenmeyer flasks of 125 cc. capacity, each containing 8 cc. of dissimilated yeast suspension (strain C1) prepared in the usual way, with galactose and water to make a total volume of 16 cc., were shaken in a water bath at 30.2°C. With this preparation, adaptation sets in soon after 90 minutes. Consequently, flasks were

removed at 90 minutes for the preadaptive period and at 180 minutes for an examination of the postadaptive period. The contents were quantitatively washed into large centrifuge tubes containing 2 cc. of 10 N H_2SO_4 to stop the reaction and precipitate the proteins. After centrifugation and removal of the supernatant fluid, the residues were washed once on the centrifuge with dilute acid and the washings added to the supernatant. The latter was made up to a definite volume in a volumetric

TABLE VI

Carbon Balance for Galactose Utilization

The results are recorded in milliequivalents of the compounds analyzed and their carbon equivalents. See text for further details.

Compounds	Products formed	Carbon atoms in products	Compounds disappeared	Carbon atoms converted
Preadaptive (0 to 90 min.)				
	m. eq.	*m. eq.*	*m. eq.*	*m. eq.*
Galactose......................	—	—	0.050	0.300
Polysaccharide (as hexose)........	0.028	0.168	—	—
O₂.............................	—	—	0.058	—
CO₂............................	0.061	0.061	—	—
Alcohol........................	0.019	0.048	—	—
Volatile acids...................	—	—	0.025	0.050
Non-volatile ether-soluble acids....	0.003	0.006	—	—
Total........................		0.283		0.350
Post-adaptive (90 to 180 min.)				
Galactose......................	—	—	0.222	1.332
Glycogen......................	0.136	0.816	—	—
O₂.............................	—	—	0.172	—
CO₂............................	0.244	0.244	—	—
Alcohol........................	0.076	0.152	—	—
Volatile acids...................	—	—	—	—
Non-volatile acids...............	0.158	—	0.006	0.012
Total........................		1.212		1.342

flask for further analysis according to the procedures described in the section on methods. The residue of yeast cells was analyzed for polysaccharides according to a modified Pflüger method (22).

The results of such balance experiments are presented in Table VI in terms of milliequivalents of products formed and compounds disappeared. Aside from the components specifically mentioned in the table, it might be noted that qualitative tests for pyruvic acid on aliquots of the original solution and the various fractions by the sodium nitroprusside test (11) were completely negative. Further, the fractionation procedure employed determines alcohol and

aldehyde together, hence qualitative tests for acetaldehyde by Rimini's modification of the method of Simon (23, 24) were applied to the original solution and to the various fractions with uniformly negative results. Since an ether extraction was resorted to, the possibility exists that the ether might have carried over acetoin or diacetyl. These were therefore tested for by the modified Voges-Proskauer procedure (25), again with negative results.

The figures in Table VI represent *net* changes in the corresponding products. The first two columns list those which show a net increase; the last two, a net decrease. An examination of the preadaptive portion reveals that 0.350 milliequivalent of carbon atoms disappeared during the preadaptive period. Of this, 0.283 milliequivalent or 81 per cent was recovered in the carbon fractions examined. The recovery of 81 per cent of the carbon disappearing is fairly satisfactory, considering that no determinations were performed to detect changes in the lipoid storage of the cells or non-volatile ether-insoluble materials such as phosphate esters. It is evident that a little more than half of the galactose utilized in the preadaptive period can be accounted for by the increase in the polysaccharide fraction. However, the oxygen consumption still amounts to only about half of the theoretical for complete oxidation of the galactose not stored as polysaccharide (0.022 milliequivalent of galactose should result in the consumption of 0.132 milliequivalent of oxygen, as against 0.058 milliequivalent actually consumed during this period). In view of the fact, however, that we are accounting in our over-all balance for only about 80 per cent of the disappearing carbon, this discrepancy may well be explained by the galactose entering some of the unanalyzed fractions.

The only anomaly in the preadaptive phase is the appearance of some alcohol. This could be accounted for on the supposition that adaptation actually comes somewhat earlier than 90 minutes. More probable, however, is that it arose from the reduction of some volatile acid component by hydrogen donors remaining in the cell at the beginning of the experiment or formed during the galactose metabolism. In this connection it will be noted that 0.05 milliequivalent of volatile acids disappeared during the incubation period which corresponds very well to the 0.048 milliequivalent of alcohol which was formed. Furthermore, the R.Q., both on an over-all basis, as is seen from the table, as well as by the continuous manometric determination during the 90 minute period of the experiment, remained close to unity. This would be difficult to understand if the alcohol arose from the metabolism of the galactose, but would be consistent if the former is formed from the volatile acids.

For purposes of comparison similar data for the postadaptive period from 90 to 180 minutes have been included in the table. The carbon recovery here is better than that obtained in the preadaptive period, 91 per cent of the galactose disappearing being accounted for. This would seem to indicate that postadaptively the galactose does not accumulate as much in the unanalyzed

fractions as is the case in the preadaptive period. Again, as before, the polysaccharide stored represents in terms of carbon about 60 per cent of the galactose which has disappeared. The difference between the oxygen consumed and CO_2 produced is 0.072 milliequivalent, in good agreement with 0.076 milliequivalent of alcohol produced.

The results obtained indicate that galactose cannot only be oxidized in the period before it can be adaptively fermented, but may also be stored in very appreciable quantities as polysaccharide as well as other intermediates.

The Kinetics of Galactose Oxidation

The ability of unadapted cells to oxidize galactose in the absence of a previous contact would argue the existence of a preformed enzyme or enzyme system, the specificity of which permitted combination with galactose. Presumably, however, the affinity of such an enzyme for a substrate like galactose would not be as great as that for the endogenous substrate it normally catalyzes. The fact that, in the competitive interaction between galactose and the endogenous polysaccharide during the preadaptive period, it is the latter which is the the preferred substrate would support such a contention.

It seemed possible that an examination of the response of the respiratory rate to increasing concentrations of galactose might reveal further information on this question. It would at any rate provide an estimate of the order of magnitude of the dissociation constant of the enzyme-substrate complex. Experiments designed to obtain such information were performed. As in all previous instances where it was desired to study the oxidation of galactose, well dissimilated cultures were used, both to lower the endogenous respiration and to extend the preadaptive period.

Fig. 9 gives a typical rate-concentration curve of an unadapted culture oxidizing galactose. For purposes of comparison there are included similar curves for an adapted culture oxidizing in one case galactose and in the other glucose. It is immediately evident that the nature of the response of the respiratory rate in an unadapted culture to increasing concentrations of galactose is entirely different from that of an adapted one oxidizing either this hexose or glucose. A much higher concentration is required in the unadapted culture in order to arrive at the maximal rate of respiration. Unlike the adapted culture, the unadapted one responds over a very wide range of concentrations to increasing amounts of galactose. It is seen that, comparatively, there is relatively little difference in the behavior of the adapted culture with galactose and glucose insofar as concerns the concentration necessary to arrive at the maximal activity.

The behavior of the unadapted culture is consistent with the interpretation that the enzyme involved has a relatively low combining capacity for galactose. Measurements of the dissociation constant, according to the procedure of

Lineweaver and Burk (26), would tend to bear this out. It was found that galactose being oxidized by an adapted culture gave a value of 0.0045. The same culture oxidizing glucose yielded a value of 0.0025. However, an unadapted culture oxidizing galactose resulted in a dissociation constant of 0.0385 which indicates a more than tenfold difference between the combining capacities of adapted and unadapted cultures for galactose.
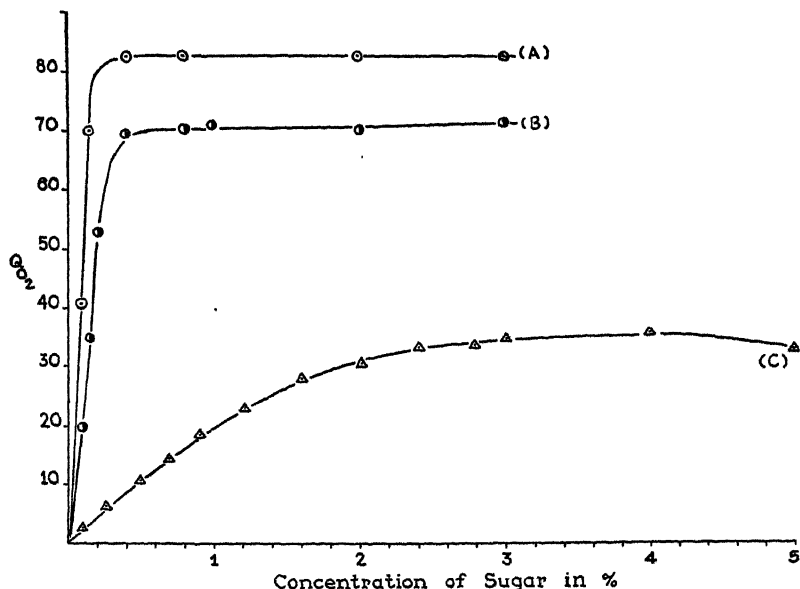

FIG. 9. Q_{O_2}-concentration curves; (A) glucose; (B) galactose with an adapted culture; (C) galactose with an unadapted culture. Each point is the average of closely agreeing duplicate determinations, and represents the steady rate for the corresponding concentration. This rate is always reached within 10 minutes after addition of the sugar to the yeast suspension.

The Oxidation of Sugars by Non-Adaptable Strains

A question which arises in connection with the ability of unadapted yeast cells to oxidize galactose is whether this ability is necessarily connected with the ability to adapt to their fermentation. It is therefore of interest to report some observations which answer this in the negative.

Strains are known which will not adapt to galactose fermentation even when incubated with this sugar in a complete medium for long periods of time. Others can give rise to mutants which on incubation with galactose will adapt to its fermentation. Table VII records the results on the measured respiratory rates when galactose is added to a series of non-adaptable strains. All these

experiments were carried out with non-dividing suspensions which rules out any possibility of mutation and subsequent selection. It is important to note further that the stimulation observed in these cases on the addition of galactose was immediate.

It is evident that these non-adaptable strains possess the capacity of oxidizing the galactose although they cannot acquire the ability to ferment. It may be worth noting that, in all the adaptable and non-adaptable strains tested thus far, no exception has been found in this capacity to oxidize the galactose. Clearly, while this physiological characteristic may be necessary for adaptation to occur in the absence of other energy sources, by itself it does not guarantee that adaptation will occur. It merely furnishes a condition under which adaptation is possible if other genetically controlled factors are present.

TABLE VII

The Effect of Adding Galactose on the Respiration of Non-Adaptable Yeast Strains

Yeast	Q_{O_2} before addition	Q_{O_2} after addition	R.Q. after addition
Schizosaccharomyces pombe	2.3	20.3	1.12
	1.8	22.9	1.00
	4.9	21.0	1.03
Schizosaccharomyces octosporus	2.0	22.0	0.98
	2.4	19.5	1.03
	3.9	24.6	1.08
Saccharomycodes ludwigii	1.0	10.4	1.00
	0.7	15.6	1.04
	0.4	12.1	1.08

It is interesting to note that normally adaptable strains can be treated in such a way that they are incapable of adapting. It has been shown (7) that length of dissimilation increases the adaptation time. If dissimilation is carried on for over 20 hours, adaptation does not occur within 48 hours. Despite this, such cells are perfectly well able to oxidize the galactose during the 48 hour period in which they show themselves unable to adapt to its fermentation.
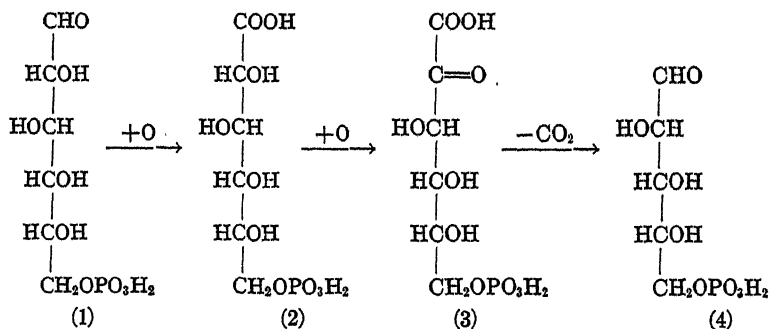
DISCUSSION

(a) General Biochemical Nature of the Oxidation of Galactose

Previous workers have offered evidence for the existence of a purely aerobic pathway for carbohydrate breakdown. Among these may be cited Loebel's (27) study of the oxidation of fructose by brain tissue, and Barker, Shorr, and Malam's (28) investigation of the continued respiration in the presence of IAA, which confirmed similar findings by Lundsgaard (14). In bacteria

Barron and Friedemann (29) present striking evidence in the form of some bacterial species which cannot ferment glucose but have no difficulty oxidizing it.

The demonstration of a purely aerobic metabolism of galactose by unadapted and unadaptable cells adds another piece of evidence to the collection of evidence against the so called "unitary hypothesis" (cf. Burk, 30) of the relation between fermentation and aerobic oxidation. The galactose differs from glucose at the fourth carbon. The trioses derived from galactose would hence be identical with those which are formed from glucose. Consequently, a cell possessing, as all unadapted cells examined here do, the full complement of enzymes necessary for fermentation of glucose could not possibly distinguish between the triose phosphate formed by the splitting of glucose and that yielded by the cleavage of galactose. The pathway of galactose oxidation by unadapted cells must therefore diverge before the 3-carbon stage is reached; for, if it did not, the ability to oxidize would have to be accompanied by the capacity to ferment this hexose. This divergence at higher levels is further supported by the investigation of Reiner (31) into the phosphate changes in the preadaptive period. It was found that phosphopyruvic acid phosphate falls markedly during the preadaptive oxidation, indicating an absence of regeneration of this compound during this period.

Since there is no evidence that galactose induces this other pathway, it is evident that it preexists functionally in normal unadapted cells. The data presented here would indicate that it represents the metabolic mechanism employed by the cell for the oxidation of its reserves. What the details of the pathway are cannot as yet be stated. Dickens (32, 33) and Lipmann (34), who have both studied the oxidation of hexose monophosphate, have proposed a scheme for the normal purely aerobic oxidative degradation of glucose which may be summarized as follows:

$$
\begin{array}{ccccccc}
\text{CHO} & & \text{COOH} & & \text{COOH} & & \\
| & & | & & | & & \\
\text{HCOH} & & \text{HCOH} & & \text{C=O} & & \text{CHO} \\
| & & | & & | & & | \\
\text{HOCH} & \xrightarrow{+O} & \text{HOCH} & \xrightarrow{+O} & \text{HOCH} & \xrightarrow{-CO_2} & \text{HOCH} \\
| & & | & & | & & | \\
\text{HCOH} & & \text{HCOH} & & \text{HCOH} & & \text{HCOH} \\
| & & | & & | & & | \\
\text{HCOH} & & \text{HCOH} & & \text{HCOH} & & \text{HCOH} \\
| & & | & & | & & | \\
\text{CH}_2\text{OPO}_3\text{H}_2 & & \text{CH}_2\text{OPO}_3\text{H}_2 & & \text{CH}_2\text{OPO}_3\text{H}_2 & & \text{CH}_2\text{OPO}_3\text{H}_2 \\
(1) & & (2) & & (3) & & (4)
\end{array}
$$

The first stage is a formation of the hexose-6-phosphate (1) and the oxidation of this to the 6-phosphohexonate (2), then to 2-keto-phosphohexonate (3), and the decarboxylation of the latter to a pentose phosphate. This scheme is based on the observations by Warburg, Christian, and Griese (35) that the hexose-

6-phosphate can be oxidized to the corresponding hexonate by enzyme preparations from yeast. Lipmann (34) obtained further evidence from manometric studies that the hexonate could oxidize to the pentose *via* the 2-keto-phospho-hexonate. Dickens (33), using Lebedew preparations plus purified coenzyme II, confirmed these findings by isolating the phosphoketohexonic acid and a phosphopentonic acid as their barium salts.

The direct application of this scheme without modification to the preadaptive oxidation of the galactose is not possible for several reasons. One would have to assume that an enzyme existed in the cell which could phosphorylate galactose in the 6 position. The recent work of Kunitz and MacDonald (36) rules out the possibility that hexokinase, the enzyme normally involved in this kind of reaction, can effect it with galactose. Furthermore, Grant (37) has shown that a preparation from galactose-adapted yeast, which could ferment galactose, failed to ferment synthetically prepared galactose-6-phosphate. A fully adapted cell would presumably contain all the components of an unadapted one plus those it gained as a result of the adaptation. Since galactose-6-phosphate is not a metabolizable intermediate for an adapted cell, it seems unlikely that it would be one for an unadapted cell.

In looking for the mechanism of galactose oxidation, the relation between the galactose oxidation and the endogenous fermentation should be borne in mind. The data on this problem can at present be best explained by supposing that the galactose is oxidized through the same metabolic mechanism which the cell employs in using its polysaccharide reserves. We may summarize the evidence leading to this conclusion as follows:—

1. Both have an R.Q. of unity and a vanishingly small anaerobic CO_2 production.

2. The behavior towards KCN of the O_2 consumption and of the aerobic CO_2 production in both types of respiration is qualitatively the same.

3. IAA in concentration sufficient to inhibit fermentation does not affect O_2 production of either the endogenous respiration or of the preadaptive oxidation of galactose.

4. The response of the O_2 consumption to addition of galactose at various levels of the endogenous respiration of a dissimilating suspension indicates the existence of a competitive interaction between them.

5. Rate of galactose assimilation increases on exhaustion of the reserves by previous dissimilation.

There are two possible mechanisms which would account for the striking similarity between preadaptive galactose oxidation and the endogenous respiration.

1. Galactose may be converted to polysaccharide before being oxidized. This would be a variation of the mechanism proposed by Willstätter and Rohde-wald (38) for the utilization of glucose and maltose.

2. That part of the galactose which is oxidized may not pass through polysaccharide but may have at least one rate-limiting step in common with the endogenous polysaccharide in their oxidative pathway.

It is not possible to decide on the precise mechanism involved here on the basis of the evidence as yet available, and it is further conceivable that both of the above may be acting as factors in the preadaptive oxidation. In this connection it should be noted that Kosterlitz (39) found that adapted yeast could utilize galactose-1-phosphate. If unadapted yeast were able to phosphorylate galactose but not able to isomerize it to the corresponding glucose ester, it would have available a close analogue of glucose-1-phosphate, which is the normal substrate of polysaccharide formation. This may explain the capacity of unadapted cells to store polysaccharide during the preadaptive period. The polysaccharide stored by adapted yeast fermenting galactose has, it is true, been found to be a polymer of glucose, and not of galactose. However, the polysaccharide formed preadaptively has never been examined in this way. In favor of the possibility that the polysaccharide formed preadaptively differs from that formed postadaptively is the sharp increase in polysaccharide storage observed after adaptation (see Table VI). This would be reasonable if glucose-1-phosphate could be formed after adaptation but not before, so that the substrate of normal glycogen formation would be available postadaptively. Otherwise, it is not easy to see why acquisition of the ability to ferment galactose should also produce a four- or fivefold increase in the ability to form polysaccharide from it. In any case, it is evident that the identification of the polysaccharide formed during the preadaptive oxidation of galactose could provide an important clue for the elucidation of the biochemical details involved.

(b) Implications of the Preadaptive Oxidation for the Adaptive Process

As was pointed out in the introduction, the existence of this preadaptive oxidation of galactose resolves the problem of how cells can adapt to galactose aerobically even though they have exhausted their endogenous reserves. Evidently the oxidation of the galactose can supply the necessary energy. We have here a situation where one and the same compound supplies both the energy and the stimulus required for a change in the enzymatic constitution of a cell.

Apparently the fermentative utilization of a carbohydrate imposes greater restrictions of specificity than its aerobic metabolism. No enzymatic modification is required for the latter pathway. Whether this is a general property of all adaptations involving carbohydrate substrates it is impossible to say. Preliminary data indicate that the same phenomenon exists in lactose adaptation by B. coli and in maltose adaptation in yeast.

The fact that the combining capacity for galactose as measured by the rate-concentration curves is greater with adapted cells than with unadapted cells raises certain questions concerning the nature of the adaptation. This fact might be interpreted to mean that the adaptive process is a consequence of

increasing the specificity of some preexisting enzymatic component which already possesses some capacity for combining with the adapting substrate. Such an interpretation would be consistent with Monod's (40) hypothesis of adaptation, which assumes that adaptation consists in increasing the specificity of some relatively non-specific protein precursor under the influence of the combining substrate. While such phenomena may take place and play a rôle in enzymatic adaptations, it seems difficult to explain the observations presented on this basis alone.

If this specificity modification were the only process involved, it would be expected that as a result of the adaptation a rise in Q_{O_2} would be the only thing observed. While such an increase in respiration in the presence of galactose is attained, by far the most unique characteristic of the adaptation is the appearance of the capacity to ferment the galactose. It would be difficult to argue that an increase in specificity of the enzyme involved in the oxidation of the galactose should result in the appearance of the capacity to ferment this hexose. This conclusion is made even more unlikely by the fact that the rise in Q_{O_2} invariably follows the appearance of the adaptive enzyme activity and never precedes it. This is seen in Figs. 1, 2, and 3, in which it may be observed that the rise in the rate of oxygen consumption always occurs after the CO_2 curve has diverged to give an R.Q. above 1, indicating aerobic glycolysis and hence adaptive enzyme activity. While such a change in specificity cannot be excluded, it seems likely that the rise in Q_{O_2} following adaptation is in part at least a result of the accumulation of oxidizable intermediates, as a result of the onset of the adaptive metabolism. This would explain the time relations of the two phenomena.

In any event it seems clear that the adaptation results in the appearance of an enzymatic activity which is essential for the anaerobic utilization of the galactose but not for its aerobic metabolism.

SUMMARY

A preadaptive purely aerobic utilization of galactose by yeast cells has been demonstrated. Hence, the adaptation by yeast to galactose is not to its utilization *per se*, but specifically to its metabolism by a glycolytic mechanism. An examination of this preadaptive oxidation of galactose reveals that it has many characteristics in common with the endogenous metabolism of yeast. Included among these are the similarities of the R.Q. values and the response of the Q_{O_2} and $Q_{CO_2}^{O_2}$ to KCN and iodoacetic acid. Further, a competitive interaction appears to exist between the endogenous respiration and the preadaptive oxidation of the galactose. The latter can replace the endogenous respiration as a source of energy for the adaptation to the fermentation of the galactose. Carbon balance studies of the galactose oxidation revealed that polysaccharide could be formed as a result of this metabolism during the preadaptive period.

Non-adaptable cells were also found to possess the capacity to oxidize galactose in the complete absence of any ability to metabolize it anaerobically.

The significance of these findings for the biochemistry and physiology of the adaptation is discussed.

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THE BIOSYNTHESIS OF C13 COMPOUNDS

I. The Biosynthesis of C13-Labeled Starch*

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INTRODUCTION

Although a rather extensive use has been made recently of the isotopes of carbon in metabolic studies of bacteria and fungi, only a few papers have appeared dealing with similar phenomena in higher plants. Ruben, Hassid, and Kamen (1), who were among the first to employ these isotopes in metabolic work, used C^{11} as a tracer in an attempt to determine the first product of photosynthesis in barley. Smith and Cowie (2) attempted to determine by means of C^{11} the relation of the dark uptake of carbon dioxide to the photosynthetic process in sunflower leaves. Belkengren (3) timed the appearance of labeled carbon in fractions of various seed plants after short periods of photosynthesis in an atmosphere containing $C^{13}O_2$. Rates of translocation were measured by Rabideau and Burr (4) in the bean plant by use of C^{13}. No chemical fractions were isolated.

None of these investigations has had as its main objective the use of the green seed plant as a means of biosynthesis.

The purpose of this series of studies is to explore the possibilities of biosynthesis of compounds suitable for metabolic studies, using $C^{13}O_2$ as the starting material and the photosynthetic process in the green plant (or chemosynthesis by autotrophic bacteria) as a means of initial synthesis. While this will result in mass labeling of the compounds so produced, rather than specific radical synthesis which can be accomplished by chemical means, it offers the advantage of the production of a large variety of compounds which cannot be readily produced by the latter process.

Many problems are apparent at the outset. Among the more important of these are the following: (a) devising specific techniques to minimize or obviate dilution of the usable products by C^{12} compounds already present in the cells of the plant; (b) either attaining high yields of these desired compounds or limiting dilution of the non-usable fractions to such an extent that they can be incinerated and their C^{13} recovered for re-use, and (c) isolating the desired compounds in a purified state.
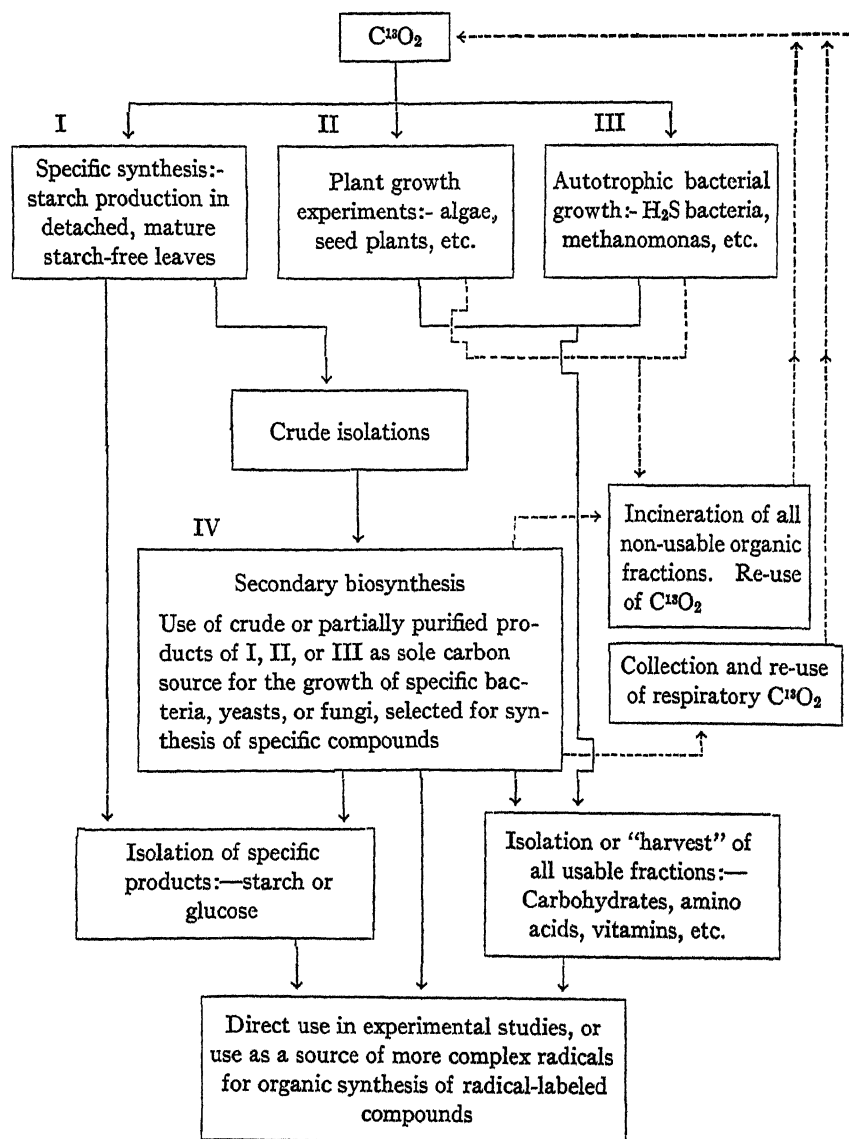
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FIG. 1. Schema for biosynthesis with organisms of various types for production of specific compounds containing C^{13}.

Several alternative methods of approach are possible, each possessing specific advantages as well as drawbacks restricting its usefulness. An over-all summary of some potentially promising lines of attack is presented in Fig. 1.

The most direct approach is *via* short-term experiments as outlined in No. I in Fig. 1. This method aims at the production of some specific compound as a relatively direct result of the photosynthetic reaction. The main body of this paper summarizes the procedure and results of an experiment of this type. An attempt was made to secure C^{13}-labeled starch with the minimum of dilution by preformed C^{12} compounds. The approach used is based on the fact that the excess carbohydrate, formed in a leaf carrying on photosynthesis at a high rate, is temporarily stored as starch in the chloroplast in which it is synthesized.

Other approaches are outlined briefly in this figure which include growing plants throughout their growing cycle (II, III, and IV) using isotopic CO_2 as a sole carbon source (II and III) or products from these as a source of carbon for secondary biosynthesis (IV). Suggestions for recovery and re-use of the carbon contained in non-usable products are included in the figure.

The results presented in this communication represent the initial step in a general project based upon this over-all plan.

Experimental Procedure

After a series of exploratory tests, leaves of *Phaseolus vulgaris*, variety Burpee's Stringless Green Pod, were selected as suitable experimental material. While satisfactory results were obtained with them, a study of other leaves is contemplated. Mature leaves were used to reduce complications introduced by growth, the leaves were subjected to a period of darkness to deplete them of the starch already present in the chloroplasts, and they were detached from the plant just before being placed in the photosynthesis chamber to obviate translocation loss after the experiment was begun.

1. *Preliminary Experiments and Their Results.*—Prior to making a final C^{13} run, a series of experiments was conducted with the aim of determining the following variables:

(*a*) The time required by bean leaves to become completely depleted of starch. Using intact plants, it was found that it required between 70 and 90 hours in darkness, depending on the initial condition of the leaves, to insure complete starch depletion of the chloroplasts in the mesophyll.

(*b*) The optimum time for the duration of the experiment. After being subjected to starch depletion, some of the leaves showed a tendency to become yellow if they were kept in the experimental chamber for a time much in excess of 48 hours. Accordingly, experiments were limited to this period of time. During these 2 days the quantity of leaves which could be readily accommodated in the chamber (about 25 gm.) was capable of utilizing practically all of the CO_2 in an initial gas mixture of 10 to 12 per cent CO_2 in air,—approximately 50 to 60 millimoles of CO_2.

(*c*) The respiratory rates of normal and starch-depleted leaves. The respiratory rates of detached bean leaves in both conditions were determined in a

separate experiment in which weighed quantities of leaves were placed in closed chambers in ordinary air in the dark and the rate of CO_2 evolution measured by making several analyses of the enclosed gas over a period of 66 hours. The respiratory rates proved to be very constant during this time interval. The rates of CO_2 evolution per hour per gram of fresh weight showed no significant difference in the two lots tested, being 0.0042 mM for the undepleted leaves, as compared to 0.0040 mM for the starch-depleted series.

2. Preparation of Plant Materials.—Plants were grown in pots out of doors until 5 weeks old. They were beginning to bloom, and each plant bore several adult trifoliate leaves, which were the type used in the experiment. The potted plants were removed to an indoor darkroom to bring about starch depletion. After 90 hours of darkness, a small piece of each leaf intended for use in the experiment was removed, boiled in 70 per cent alcohol, stained in 1 per cent iodine-potassium iodide solution, and examined microscopically. All leaves were found to be entirely free of visible starch grains, except in the guard cells of the epidermis, where fairly large starch granules were still present. One leaflet from each of six different leaves was removed and stored frozen for a subsequent quantitative determination of starch and soluble sugars initially present. The leaves to be used were severed from the plants at the base of the petiole immediately before setting up the experiment. Fig. 2 is a diagram of the photosynthetic chamber employed.

The $C^{13}O_2$ generator was prepared by placing a weighed quantity (54.01 mM) of $BaC^{13}O_3$ in a porcelain evaporating dish directly beneath the tube of the dropping funnel, and introducing 100 ml. of 20 per cent lactic acid into the reservoir of the dropping funnel.

In setting up this experiment, the vases were filled with CO_2-free water, and the leaves were arranged around the sides of the jar, using narrow strips of Scotch tape at the tips of the leaflets to hold them in place for maximum exposure to light. All of this and subsequent operations were carried out as rapidly as possible in subdued light. After all preparations were completed, the cover was clamped in place and the juncture between the jar rim and rubber gasket liberally painted with shellac to insure a perfect seal. CO_2-free air was then flushed through the chamber for 1 hour, the chamber was evacuated to approximately 50 cm. Hg, and $C^{13}O_2$ generated by allowing the lactic acid to flow slowly into the dish of $BaC^{13}O_3$. The chamber was immersed in a water bath with glass sides at approximately 24°C. and allowed to stand under reduced pressure for 1 hour to facilitate complete evolution of CO_2. Finally the internal pressure was equalized to atmospheric by allowing CO_2-free air to enter through one of the stopcocks.

Before removing the initial gas samples for analysis, a 100 ml. syringe was attached to the Luer stopcock, and the gas in the chamber was thoroughly mixed by 30 cycles of rapid filling and emptying of the syringe. The gas samples for analysis were removed in smaller syringes, each provided with a stopcock. In the removal of all subsequent samples, the additional provision was made before mixing, of first adjusting the chamber to atmospheric pressure by the introduction of CO_2-free air.

Two banks of lights, each consisting of four 20-W fluorescent bulbs were placed outside the water bath, approximately 4 inches to either side of the chamber. These lights were turned on immediately after the removal of the initial gas samples referred to above.

Total CO_2 and O_2 content of the gas was determined in duplicate with the Scholander

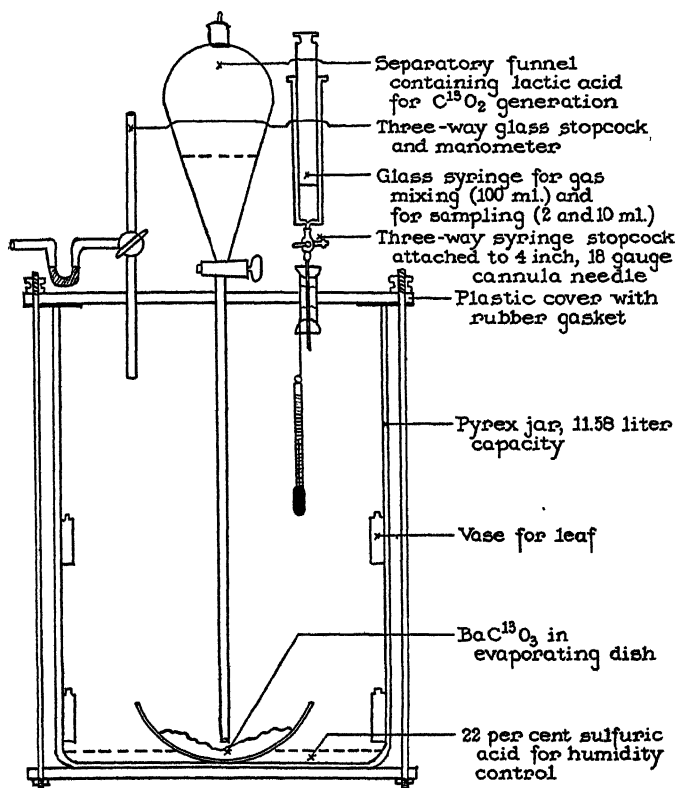FIG. 2. Photosynthetic chamber.

Labels in figure:
- Separatory funnel containing lactic acid for $C^{13}O_2$ generation
- Three-way glass stopcock and manometer
- Glass syringe for gas mixing (100 ml.) and for sampling (2 and 10 ml.)
- Three-way syringe stopcock attached to 4 inch, 18 gauge cannula needle
- Plastic cover with rubber gasket
- Pyrex jar, 11.58 liter capacity
- Vase for leaf
- $BaC^{13}O_3$ in evaporating dish
- 22 per cent sulfuric acid for humidity control

gas analyzer (5), using approximately a 1 ml. sample for each determination. The C^{13} content of the gas at the beginning and end, as well as of all of the products of the experiment was determined by the mass spectrometer. Complete data on the gas analyses throughout the experiment are presented in Table I.

The experiment was terminated at the end of 48 hours. As soon as the lights were turned off, the chamber was flushed for 1 hour with CO_2-free air, and all residual CO_2 was absorbed in a train of NaOH vessels attached to the exit stopcock. The carbonate was converted to $BaCO_3$, filtered under CO_2-free conditions, dried, weighed, and analyzed.
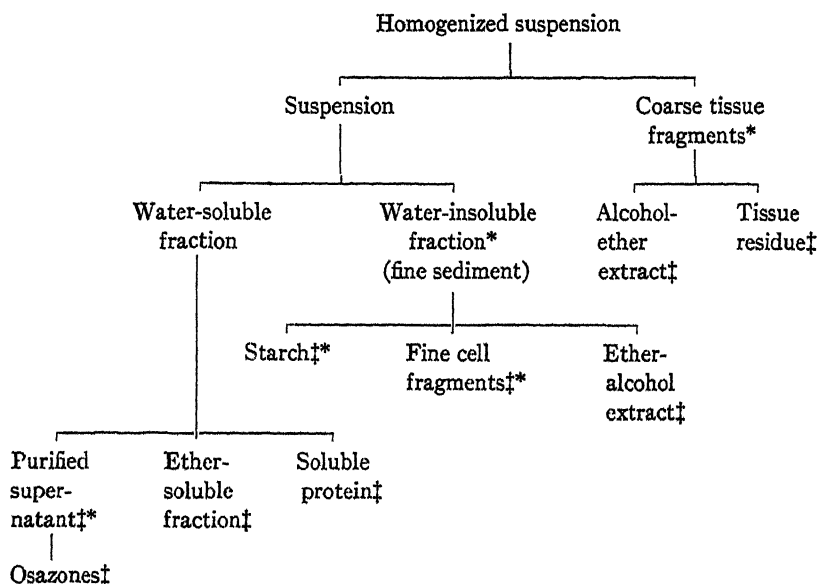
The chamber was opened, and the leaves were removed and rechecked for fresh weight. The total weight was 27.8 gm. They were divided into two lots, one lot of

TABLE I

Gas Analysis

Summary Table

Phaseolus vulgaris leaves (27.8 gm. wet weight) were depleted of carbohydrate and illuminated for 48 hours in a closed chamber of 11.58 liter capacity. CO_2 was generated from $BaCO_3$ with lactic acid in an atmosphere of CO_2-free air. CO_2 and O_2 analyzed with Scholander micro gas analyzer.

Time	CO_2	O_2	C[13] excess
hrs.	*per cent*	*per cent*	*per cent*
0	11.2	16.7	7.26
24	5.45	22.25	—
41.5	1.5	26.1	—
47	0.5	27.0	—
48	0.1	—	2.66*

* This figure is too low since some contamination with atmospheric CO_2 occurred during the collection of the sample.

Homogenized suspension

Suspension   Coarse tissue fragments*

Water-soluble fraction   Water-insoluble fraction* (fine sediment)   Alcohol-ether extract‡   Tissue residue‡

Starch‡*   Fine cell fragments‡*   Ether-alcohol extract‡

Purified supernatant‡*   Ether-soluble fraction‡   Soluble protein‡

Osazones‡

* Analyzed for carbohydrate.
‡ Analyzed for C[13].

FIG. 3. Fraction of *Phaseolus vulgaris* leaves for C[13] analysis. 10 gm. aliquot of fresh leaves (dry weight, 1.709 gm.).

10 gm. and the second lot of the remaining 17.8 gm. They were placed in large test tubes, and stored in a refrigerator in a frozen state.

The water remaining in the vials was transferred to a bottle of $Ba(OH)_2$ under CO_2-free conditions. The precipitate was recovered by filtration, dried, weighed, and analyzed.

3. *Preparation of Plant Material for Analysis.*—A summary flow sheet of fractionation procedure is presented in Fig. 3. The points at which samples for carbohydrate and for C^{13} analyses were removed are indicated. All of the initial steps in the procedure, including centrifugation, washing of the sediment fraction, and deproteinization of the soluble fraction were carried out in a cold room with reagents maintained at approximately 5°C. to minimize enzyme action.

The 10 gm. sample of frozen leaves was processed in a Waring blendor for 5 minutes in 90 ml. of distilled water and then strained through a cloth of fine mesh. The residual tissue fragments were resuspended in 50 ml. of water and reprocessed in the blendor for 5 minutes and restrained. This was repeated once more, and the entire liquid suspension combined. Microscopic examination of the coarse tissue fragments remaining indicated that they consisted principally of vascular and epidermal fragments. No intact mesophyll cells were observed, and the only visible starch-containing cells in this fraction were the guard cells in the epidermal fragments. Very few of these appeared to be destroyed by the blendor treatment, and it is assumed that the major part of the epidermal tissue and therefore, the guard cell starch, remained in the coarse tissue fragment fraction. Water was used in preference to the 70 per cent alcohol usually employed because it was found that the alcohol interfered with later purification of the starch by sedimentation. The suspension contained practically all of the chloroplast starch present in the mesophyll cells of the leaves, chloroplast fragments, coagulated protoplasm, and other fine particles of insoluble material. It contained also all soluble cell constituents, including sugars, soluble proteins, organic acids, other miscellaneous organic constituents, and inorganic salts. This initial fraction was further separated by centrifugation into a total water-soluble fraction and a water-insoluble fine sediment fraction. The latter was washed and centrifuged twice with 25 ml. of water for each washing. The washings were added to the water-soluble portion.

These procedures had now separated the plant material into three fractions which are designated as the coarse tissue fragment fraction, the water-insoluble fine sediment fraction, and the water-soluble fraction.

The water-insoluble fine sediment fraction, consisting chiefly of starch, coagulated protoplasmic components, and chloroplast fragments, was subjected to repeated differential sedimentations and centrifugations which eventually resulted in the separation of a starch fraction, and fine cell fragments. These fractions were extracted with alcohol and ether, resulting in three fractions, purified starch, fine cell fragments, and the combined ether-alcohol extract. A small quantity of starch was detectable by microscopic examination in the fine cell fragments.

The total water-soluble fraction was first treated with $ZnSO_4$ to precipitate soluble proteins. The zinc protein precipitate was resuspended in water and freed from zinc with H_2S. The dissolved protein was purified by repeated $(NH_4)_2SO_4$ precipitation and dialysis and dried for C^{13} analysis.

The deproteinized supernatant was rendered zinc-free and concentrated in a vacuum desiccator to about 80 ml. and extracted for 50 hours with ether in a reflux condenser to remove organic acids and other ether-soluble constituents for C^{13} analysis. Soluble sugars remained. To determine their C^{13} content, an aliquot portion was hydrolyzed and a phenylosazone prepared. (We are indebted to Dr. Peter Wenck of our staff for this preparation.)

Carbohydrate analyses were made on the various fractions as indicated by asterisks in Fig. 3. The general method of starch analysis consisted of grinding the tissue, washing the insoluble fraction free of soluble sugars, dispersing the starch by boiling the insoluble fraction in water, and then digesting for 1 hour with pancreatic amylase. Soluble protein was precipitated with lead acetate, and the deproteinized solution was subjected to acid hydrolysis to convert all sugars to monosaccharides. Sugar content was determined by the ceric sulfate method of Miller and Van Slyke (6). Soluble sugars in aqueous fractions were determined by the ceric sulfate method after deproteinization and acid hydrolysis.

The various fractions which were analyzed for C^{13} content are indicated by double daggers in Fig. 3.

RESULTS

The C^{13} content of the various isolated fractions is presented in Table II (see also Fig. 3 for isolation procedures). All quantities were computed on the basis of the total quantity of plant material used in the experiment (27.8 gm. fresh weight).

The C^{13} excess in the CO_2 taken up by the plants during the experiment was 3.884 mM, computed by subtracting 0.013 mM $C^{13}O_2$ recovered from the vase water and 0.012 mM recovered from the residual CO_2 from the 3.910 mM initially supplied as $C^{13}O_2$.

The C^{13} content of the ether-soluble fraction of the aqueous extract (0.28 atom per cent C^{13} excess) and the soluble protein fraction (0.31 atom per cent excess) indicate that very little exchange or resynthesis occurred in these fractions during the course of the experiment. The amounts of material in these two fractions were too small to allow total carbon determinations, so no data are available as to the amount of C^{13} contained in them. The remaining soluble constituents had a C^{13} excess of 2.10 atom per cent, most of which was in the soluble sugars present in the mixture (see Table IV).

Approximately one-half of the total starch initially present in the suspension (by starch analysis) was recovered in a purified state by the fractional sedimentation technique used. Microscopic examinations showed no constituents other than starch in this fraction. The alcohol-ether– soluble fraction included the plastid pigments, as well as a mixture of lipids and other water-insoluble organic materials. Since this fraction had only a C^{13} content of 0.50 atom per cent excess, no further fractionations were made. The remaining fine cell fragments included some of the smaller starch grains, which probably account for the major

portion of the C^{13} content of this fraction (2.26 atom per cent C^{13} excess). It would have been of interest to have prepared a carbohydrate-free sample of this fraction in order to determine the C^{13} content of the chloroplast and protoplasmic fragments, but no such preparation was made.

As indicated in the discussion of methods above, the coarse tissue fragments which were retained by the fine mesh cloth used for straining consisted chiefly of vascular debris and fairly large epidermal fragments (including guard cells and their contained starch). The C^{13} excess present in this fraction, 0.66 atom

TABLE II

C^{13} Excess in Fractions Isolated from Leaves of Phaseolus vulgaris

Leaves were previously depleted of starch and reilluminated. C^{13} excess in the CO_2 taken up by the leaves during the experiment was 3.884 mM.

	C^{13} excess		
	Atom	mM	C^{13} excess utilized
	per cent		*per cent*
Total suspension			
Total soluble fraction			
Ether extract..............................	0.28	—	—
Soluble protein...........................	0.31	—	—
Soluble protein-free fraction...............	2.10	0.643	16.56
Total sediment			
Alcohol-ether–soluble fraction.............	0.50	0.018	0.67
Purified starch............................	7.05	1.411	36.30
Fine cell fragments........................	2.26	0.313	8.06
Coarse tissue fragments......................	0.66	0.378	9.73
Total.......................................			71.32

per cent, would, in part, be accounted for by increase in starch and by a possible increase or exchange involving other cell constituents. These fragments were also extracted with alcohol and ether, thereby obtaining a plastid pigment-lipid fraction. The C^{13} content of this extract was 0.91 atom per cent C^{13} excess. It is to be noted that this is higher than the value for the comparable extract from the fine sediment. Since no attempts were made to isolate separate constituents from either of these fractions, no explanation of this difference is proposed.

A quantitative summary of the carbohydrates present in the leaves at the beginning and end of the experiment, as determined by anlysis, is presented in Table III. Table IV presents a quantitative summary of the C^{13} excess accounted for in the carbohydrate fractions. It should be noted that the starch

TABLE III

Carbohydrate Summary

Carbohydrate gained by leaves of *Phaseolus vulgaris* depleted of starch, then illuminated for 48 hours. Carbohydrate is computed as glucose.

	Initial		Final		Gain	
	gm.	*per cent*	*gm.*	*per cent*	*gm.*	*per cent*
Soluble sugar						
Suspension.................	0.176	0.63	0.397	1.43	0.221	126.0
Coarse tissue fragments.......	0.0	0.0	0.0	0.0	0.0	—
Starch						
Suspension.................	—*	—*	1.112	4.00	1.112	—
Coarse tissue fragments.......	0.115‡	0.42‡	0.129	0.46	0.014	12.2

* Assumed to be zero since no starch was detectable in mesophyll upon microscopic examination (iodine test).

‡ Determined in total sample, but assumed to be in the coarse tissue fragments, since starch was visible in chloroplasts of guard cells only.

TABLE IV

C^{13} Excess Summary

The amounts of starch and sugar were determined quantivatively (see Table III). All values were computed as glucose. C^{13} atom per cent excess was determined on an aliquot of the starch isolated by fractional sedimentation and on a phenylosazone prepared from the soluble sugar fraction.

C^{13} excess in CO$_2$ taken up by the leaves during the experiment = 3.884 mM.

	Gm.	C^{13} excess		
		mM	Atom	C^{13} excess utilized
			per cent	*per cent*
Suspension				
Soluble sugar.........................	0.397	0.889	6.72	22.90
Starch.............................	1.112	2.613	7.05	67.28
Total carbohydrate....................	1.509	3.502	—	90.18
Residue (coarse tissue fragments)..........	1.50	0.378*	0.66	9.73
Total...............................				99.91

* Determined on total tissue residue fraction.

synthesized during the experiment accounts for 67.28 per cent of the total C^{13} excess taken up by the leaves, while the soluble sugar accounts for 22.90 per cent making a total of 90.18 per cent of the total accounted for in these fractions. These figures were computed by combining the data obtained from

quantitative carbohydrate determinations with the C^{13} assay of purified aliquots of these fractions. The coarse tissue fragments removed by straining contained an additional 9.23 per cent of the total, thus accounting for 99.91 per cent of the C^{13} absorbed.

DISCUSSION

In this experiment, a compound which cannot be synthesized chemically has been produced with an efficiency comparable with that of a relatively simple chemical synthetic procedure. With an initial C^{13} content of 7.26 atom per cent excess in the CO_2 supplied, the starch with a C^{13} atom per cent excess of 7.05 represents a dilution of only 2.9 per cent; while the soluble sugar fraction, with a C^{13} atom per cent excess of 6.72 shows a reduction in isotope concentration of 7.4 per cent. The starch which can be isolated with little difficulty, accounts for approximately 67 per cent of the C^{13} absorbed by the plants. The soluble sugar fraction accounts for approximately 23 per cent. The latter fraction, however, has not yet been isolated.

A summary flow sheet tracing the probable fate of the carbon in the experimental system is proposed in Fig. 4. Assuming a respiratory rate of 0.004 mM of CO_2 per hour per gram of fresh weight of the leaves, as determined in a separate experiment, the 27.8 gm. used would evolve 5.66 mM of CO_2 during the 48 hour run. Adding this to the initial CO_2 supplied (53.36 mM)[1] would give a total amount of 59.52 mM of CO_2, of which the respiratory CO_2 constitutes 9.5 per cent.

It is of interest to note that the net respiratory turnover during the experiment (5.66 mM of CO_2) would require 0.94 mM (169 mg.) of hexose if this were the sole substrate. This quantity constitutes almost as much soluble sugar as was present in the leaves initially, and 42.6 per cent of the amount of soluble sugar present in them at the end. If this quantity is added to the amount present at the end of the experiment, the total soluble sugar involved would be 566 mg., and the quantity synthesized during the experiment would be 390 mg. On this basis, respiration would utilize 30 per cent of the gross amount. The fate of the pool of soluble carbohydrate initially present in the leaves bears an important relation to the amount of dilution which occurred in the final carbohydrate fractions. If a major portion of the soluble carbohydrate initially present remained in the cells unchanged, most of it would be accounted for as a dilution factor in the soluble carbohydrate fraction at the end of the experiment. Conversely, if this constituted the chief respiratory substrate during the course of the experiment, we should expect a considerable dilution of the

[1] This figure was based on gas analysis. The slight discrepancy between it and that derived from the weight of the $BaC^{13}O_3$ employed is probably due to the impossibility of measuring accurately the volume of gas in the experimental chambers.

CO_2 initially provided and consequently of all products formed from it.   The following computations may throw some light on these questions.

53.86 mм CO_2

Photosynthesis

CO_2 recovered from vase-
water
0.309 mм CO_2
4.19 atom per cent C^{13} excess
0.013 mм excess C^{13}

Respiratory CO_2‡
5.66 mм

Starch
37.07 mм C
7.05 atom per cent C^{13} excess
2.613 mм excess C^{13}

Pool of soluble sugar

Initial	Final
5.62 mм total C	13.23 mм total C
0.0 atom per cent C^{13} excess	6.72 atom per cent C^{13} excess
0.0 mм excess C^{13}	0.889 mм excess C^{13}

Non-carbohydrate fraction

Residual CO_2
0.466 mм C
2.66 atom per cent C^{13} excess
0.012 mм excess C^{13}

   * See text for explanation.
   ‡ Computed from control sample.

FIG. 4. CO_2 flow diagram.   Volume of chamber = 11.58 liters. CO_2 = 11.3 per cent of gas mixture (53.86 mм)*. C^{13} excess in CO_2 = 7.26 atom per cent (3.910 mм).

If the soluble carbohydrates initially present (0.977 mм as glucose, containing 5.87 mм of carbon) had been unused, and if then the soluble carbohydrate content had been built up to 2.205 mм by the addition of 1.227 mм of glucose containing 7.36 mм of carbon synthesized entirely from the CO_2 supplied (7.26 atom per cent C^{13} excess), the final concentration of C^{13} in this fraction at the end of the experiment would have been

$$\frac{(5.87 \times 0) + (7.36 \times 7.26)}{5.87 + 7.36} = 4.48 \text{ atom per cent } C^{13} \text{ excess}$$

which may be seen to be much lower than that obtained (6.72 atom per cent C^{13} excess). Therefore we know that a considerable amount of the original pool of soluble carbohydrate must have disappeared from the fraction.

If, on the other hand, all of the carbon in the soluble carbohydrate initially present (5.87 mM of carbon) had remained in the carbohydrate-CO_2 system and become redistributed by contributing materially to the respiratory CO_2, or had remained as carbohydrate *per se*, then the C^{13} concentration of the entire system should have been

$$\frac{(5.87 \times 0 \text{ per cent}) + (53.86 \times 7.26 \text{ per cent})}{53.86 + 5.87} = 6.64 \text{ atom per cent}$$

average C^{13} excess. This represents the maximum average C^{13} excess which would have been possible in the combined carbohydrate fractions under the conditions specified above. The figure obtained was 6.97 atom per cent excess. From these considerations we may conclude that the carbon in the initial soluble carbohydrate fraction which disappeared was not totally converted to CO_2 by respiration, nor did it all remain in the carbohydrate fraction.

The only apparent alternative appears to be that a portion of the carbohydrate initially present in the leaves took part during the experiment in some unknown synthetic process yielding non-carbohydrate products, and that the respiration of the leaves during the course of the experiment was supported principally by the primary product of photosynthesis. The small amount of dilution which did occur in this experiment was probably derived principally from the initial soluble carbohydrate fraction, both by respiration and retention as carbohydrate, but the importance of this dilution factor was materially less than was anticipated.

There was only a small amount of C^{13} present in the non-carbohydrate fractions isolated. Apparently very little resynthesis or exchange in these fractions occurred in the mature leaves used, and in the short time involved in the experiment.

SUMMARY

1. Starch, containing 7.05 atom per cent C^{13} excess has been produced in the mesophyll cells of bean leaves, starting with $C^{13}O_2$ containing 7.26 atom per cent C^{13} excess. Approximately 67 per cent of the C^{13} taken up by the leaves was determined in the starch fraction.

2. The soluble carbohydrate, containing 6.72 atom per cent C^{13} excess, accounts for approximately 23 per cent of the C^{13} taken up by the leaves. The remainder was principally in the coarse tissue fragments fraction (9.73 per cent of the C^{13} utilized).

3. The apparatus and procedures used in this experiment are described.

We are indebted to the Houdry Process Corporation of Marcus Hook, Pennsylvania, and particularly to Dr. Sidney Weinhouse for the C^{13} analyses; also to Swarthmore College for the loan of their Scholander analyzer.

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STUDIES ON THE SONIC TREATMENT OF TOBACCO MOSAIC VIRUS*

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PLATE 4

INTRODUCTION

Takahashi and Christensen (1) found that the juice of plants suffering from tobacco mosaic virus disease was rendered non-infectious when subjected to intense sonic vibrations. Stanley (2) found that the biological activity of purified tobacco mosaic virus is reduced by sonic treatment and demonstrated that the sound has little or no effect on the activity of the virus if cavitation, normally associated with strong vibrations in liquids, is supressed by a lowering of the atmospheric pressure above the liquid. Kausche, Pfankuch, and Ruska (3) found with the electron microscope that sonic treated tobacco mosaic virus samples contained more short rod-like particles than are observed in untreated samples. The sonic treatment apparently results in a breakage into shorter fragments of the long rod-like particles associated with the tobacco mosaic virus disease and offers a convenient method of studying the relation between the size of the particles and their biological activity.

In experiments described in this paper, samples of centrifugally purified tobacco mosaic virus were subjected to strong sound vibrations for varying lengths of time. The physicochemical properties of the sonic treated material were determined and the material was tested for biological activity. The virus particles as well as the fragments produced by sonic treatment were made to aggregate end-to-end and the properties of the aggregates were studied.

EXPERIMENTAL METHODS AND RESULTS

Sonic Treatment.—Solutions of tobacco mosaic virus purified by differential centrifugation by the method of Stanley (4) were sonic treated in a magneto-striction sound generator operating at 9,000 cycles per second and producing approximately 100 watts of acoustic energy. The apparatus (type R-22-1 oscillator developed by the Submarine Signal Co.) contains an effective water-cooling system so that at no time did the temperature rise above 17°C. The author is indebted to Dr. Thomas Anderson of the Johnson Foundation for the use of this apparatus and also to Dr.

L. A. Chambers, formerly of the Johnson Foundation, for the use in preliminary experiments of the magneto-striction sonic generator developed by Chambers and Flosdorf (5). It was found that both sonic generators, when tuned to maximum efficiency, delivered approximately equal energy to the liquid.

When the virus solutions were subjected to the sound vibrations, violent swirling was observed. The swirling is associated with cavitation in the liquid, which is caused by the strong sound vibrations. When cavitation is suppressed by lowering the atmospheric pressure above the liquid, the swirling disappears.

Stream Birefringence and Viscosity.—The stream birefringence of the solutions of purified tobacco mosaic virus was observed by inverting a test tube of the material
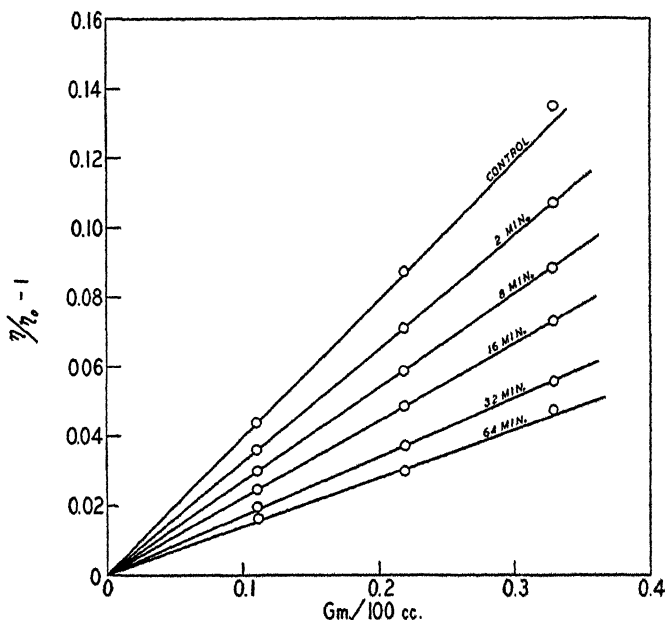
TEXT-FIG. 1. Specific viscosity as a function of concentration for purified tobacco mosaic virus sonic treated for 0, 2, 8, 16, 32, and 64 minutes.

between crossed polaroids. It was found that the intensity of stream birefringence decreased roughly exponentially with time of sonic treatment.

The viscosities of solutions of tobacco mosaic virus also decrease as sonic treatment proceeds. Solutions of centrifugally purified virus (3.28 mg./cc. in 0.1 M phosphate buffer at pH 7.0) were subjected to sonic treatment for 2, 8, 16, 32, and 64 minutes. Viscosity measurements of various dilutions of the solutions were made in an Ostwald viscometer kept at constant temperature in a water bath at 27 ± 0.005°C. The specific viscosity, $\eta/\eta_0 - 1$, where η is the viscosity of the solution, and η_0 the viscosity of the solvent, was found to be linear with concentration (Text-fig. 1).

Electron Microscope Studies.—Electron micrographs were taken of samples of purified tobacco mosaic virus subjected to sonic treatment for varying lengths of

time. Small amounts of the samples were diluted to a concentration of 0.1 mg./cc. and transferred to electron microscope screens. The screens were allowed to dry and then dipped into distilled water to remove soluble salts. In order to obtain greater photographic contrast the screens were gold shadow cast according to the method of Williams and Wyckoff (6). The samples were observed in an RCA Console Model (type EMC-1) electron microscope having a magnification of 5,800.

Typical micrographs of the control material and of material sonic treated for 16 minutes are illustrated in Figs. 1 and 2. It is apparent from the micrographs that as a result of the sonic treatment the rod-like particles of the tobacco mosaic virus are broken up into fragments which have the same circular cross-section with a diameter of 15 mμ as have the original particles.

Representative electron micrographs were enlarged photographically and the lengths of all the particles in each picture were measured in intervals corresponding to 38 mμ with a variation within 19 mμ from the mean value of the interval. This spread in measurement was taken in an attempt to overcome all the errors involved in measuring particles of these sizes. The errors have been estimated to be about 10 mμ (7). The spread taken here is sufficiently narrow to show the essential features of the results of sonic treatment. About 300 particles from each sample were counted.

Text-fig. 2 gives the results of the size distribution measurements from electron micrographs of the control material and material sonic treated for 2, 8, 16, 32, and 64 minutes. Sixty-two per cent of the particles in the untreated sample are about 280 mμ in length. The control material was obtained from Turkish tobacco plants which had been infected for 25 days with tobacco mosaic virus and was purified within a week after harvesting the plants. The sample gives a size distribution of the particles approximately that found by Oster and Stanley (8) in the contents of hair cells from diseased plants. Large deviations from this size distribution for a purified sample can usually be attributed to the method of preparation and age of the sample. Thus Sigurgeirsson and Stanley (9) found a large number of aggregates of the 280 mμ in length particles in samples which had been allowed to stand for long periods of time.

After a sonic treatment of only 2 minutes the number of rods about 280 mμ in length is considerably reduced and the number of particles about half this length is increased. On further sonic treatment the number of particles 280 mμ in length decreased exponentially with time. The number of half lengths (about 140 mμ) increased and then decreased with time and the number of one-fourth lengths subsequently increased and then decreased with time. Because of their small size, the exact number of particles of one-fourth length and shorter is difficult to determine. It is clear, however, that practically no particles are produced having lengths between 280 mμ and 140 mμ, and not many particles are produced having lengths between 140 mμ and 70 mμ.

Chemical Effects of Sonic Treatment.—It has been observed (10) that thermal

denaturation of tobacco mosaic virus results in the production of insoluble protein and the release of nucleic acid. Prolonged sonic treatment, however, evidently produced no appreciable amount of insoluble protein since there
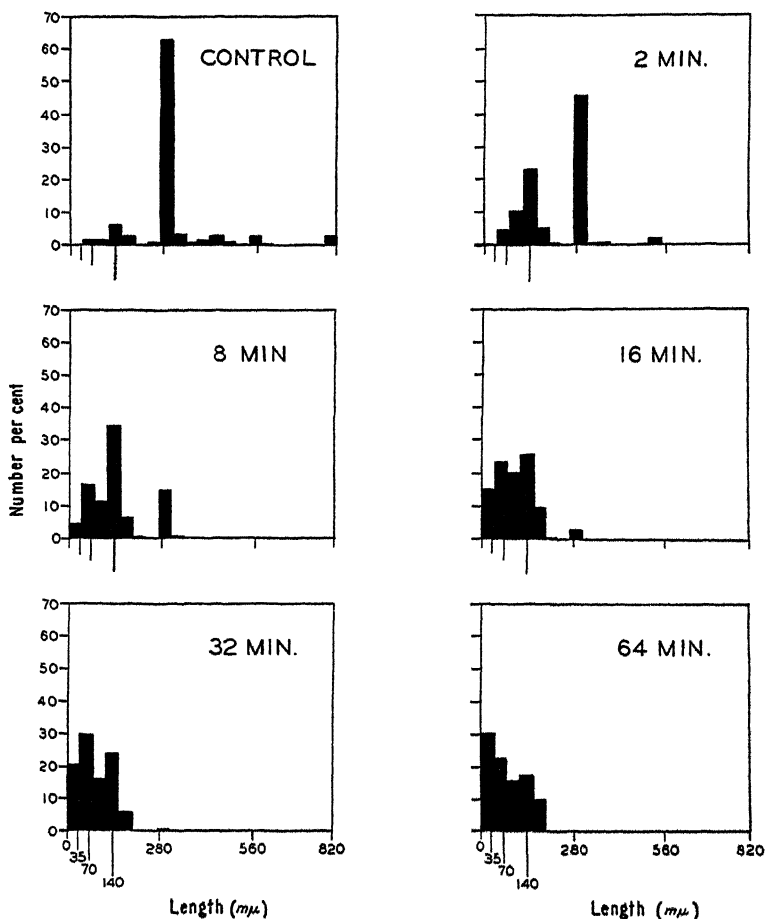
TEXT-FIG. 2. Size distributions from representative electron micrographs of purified tobacco mosaic virus sonic treated for 0, 2, 8, 16, 32, and 64 minutes (300 particles counted for each sample).

was no increase in the turbidity of the virus solutions. An attempt was made to determine whether nucleic acid was released on breakage of the particles by sonic treatment. An aqueous solution of purified tobacco mosaic virus, which had been sonic treated for 32 minutes, was brought to a concentration of 0.1 N with respect to sodium chloride. The solution was then brought to

its isoelectric point with dilute acetic acid and the precipitated material was spun for 15 minutes at 5,000 R.P.M. in an angle centrifuge. Since no nitrogen was detectable in the clear supernatant it may be concluded that no nucleic acid or other nitrogen-containing substances soluble at this pH and salt concentration was released.

The isoelectric points of many proteins are known to change when the protein is denatured. It was found, however, that the isoelectric point, as determined by a turbidimetric method (11), of sonic treated tobacco mosaic virus in distilled water was pH 3.92, which is the same as that of the untreated sample.

The immunological properties of sonic treated tobacco mosaic virus have been investigated by Dr. S. Malkiel of this laboratory (12). He found that sonic treated virus precipitated more antibody per unit weight of antigen,

TABLE I

Biological Activity of Sonic Treated Tobacco Mosaic Virus

Time of treatment	Dilution	No. of lesions (treated/control)	Relative activity
min.		.	*per cent*
0	1:500	—	(100.0)
2	1:200	1791/865	82.8
8	1:100	1265/739	34.3
16	1:10	1476/324	9.13
32	1:5	1169/743	1.57
64	1:1	450/1041	0.086

than did the untreated virus. This increase is attributed to the increased surface area of the broken-up virus particles.

It may be concluded from these observations that the chemical properties of the particles are not changed appreciably by sonic treatment.

Biological Activity.—The biological activity of the sonic treated tobacco mosaic virus was determined from the number of lesions produced by it on *N. glutinosa* according to the local lesion method of Holmes and others (13). Preliminary tests were made to determine the order of magnitude of the biological activity of the treated and of the untreated virus. The samples were diluted in phosphate buffer so that each sample would give a number of lesions on one-half of the leaf comparable to the number given by the control material, diluted 1:500, on the other half. In Table I are given the results of these tests.

In order to determine whether the shorter rods have an inhibitory effect on the larger ones, mixtures of the control and the 64 minute treated sample were made and applied to the plants. It was found that there was no appre-

ciable difference in activity which could not be accounted for on the basis of the relative activities of the constituents of the mixtures.

In Text-fig. 3 the logarithm (base 10) of the relative activity is plotted as a function of time of sonic treatment. It is seen that the activity decreases exponentially with a rate constant given approximately by $k = 0.13$ min.$^{-1}$

Aggregation of the Particles.—It is well known that various chemical agents can cause end-to-end aggregation of tobacco mosaic virus particles. The electron micrograph studies of Sigurgeirsson and Stanley (9) showed that there
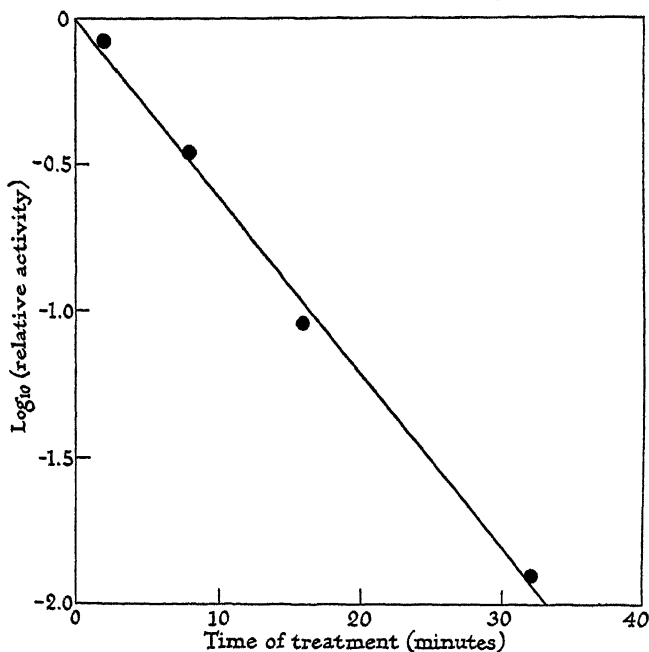
TEXT-FIG. 3. Biological activity of purified tobacco mosaic virus as a function of time of sonic treatment.

is considerable particle aggregation in expressed juice of diseased plants allowed to stand for long periods of time. Bawden and Pirie (14) have also shown that incubation of tobacco mosaic virus with trypsin or snail enzymes can cause aggregation of the particles.

A simple method for causing aggregation which does not require the introduction of extraneous protein material is the following: A solution of purified virus suspended in distilled water is precipitated by being brought to its isoelectric point (pH 3.92 in water) with 0.001 N hydrochloric acid. The sample is then incubated for 3 days at 37°C. The clear supernatant liquid is removed and the precipitate is dissolved in 0.1 M phosphate buffer at pH 7.0. This solution shows the same stream birefring-

ence as the original solution; but, if this material is now incubated for an additional 4 hours, it shows greatly increased stream birefringence.

When untreated virus particles are aggregated by the isoelectric method, the solution is highly thixotropic and the viscosity cannot be measured in an Ostwald viscometer. An electron micrograph of this material is shown in Fig. 3. The small beading along the rods and the granular background are artifacts caused by excessive exposure of the gold shadow cast film to the elec-
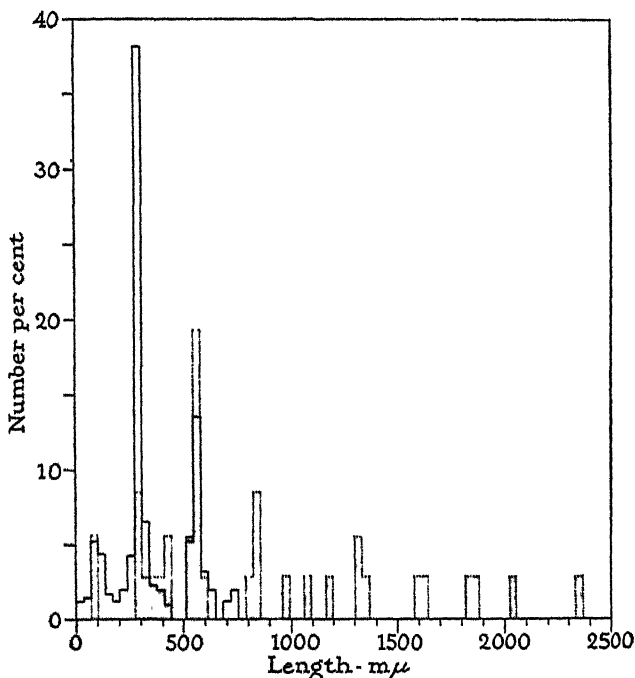
TEXT-FIG. 4. Shaded area, size distribution of aggregated tobacco mosaic virus particles (36 particles counted). Unshaded area, size distribution of the same material after strong stirring (265 particles counted).

tron beam in the microscope (15). A size distribution of the particles is shown in the shaded area of Text-fig. 4. Since the particles are very long and only a few appear on each micrograph, a size distribution was made of only 36 particles taken from a composite of four micrographs. This size distribution cannot, therefore, be regarded as having great statistical significance, but it shows that the predominant size corresponds to dimers of the 280 mμ length. The aggregated untreated virus was tested for biological activity by the lesion method and was found to be 48 per cent as active as the unaggregated material.

The long aggregated particles were broken up when the sample was subjected to strong stirring with a propeller motor-driven at about 6,000 R.P.M. The propeller

blade was 1 cm. in length and the sample was placed in a test tube 2.5 cm. in diameter kept in a cooling bath to prevent heating by the stirring. After the aggregated material had been stirred for 15 minutes, it showed a marked decrease in stream birefringence and in viscosity, and its biological activity was 92 per cent of that which it was before aggregation. The size distribution of 265 particles observed in electron micrographs of the stirred sample is shown in the unshaded area of Text-fig. 4. Thirty eight per cent of the particles are about 280 mμ in length and only 13 per cent are dimers.
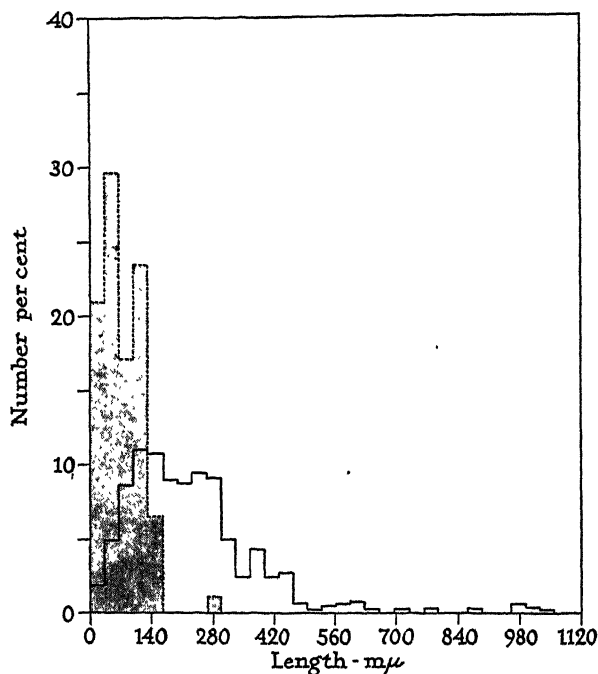
TEXT-FIG. 5. Shaded area, size distribution of particles of tobacco mosaic virus sonic treated for 32 minutes (300 particles counted). Unshaded area, size distribution of the same material after aggregation (300 particles counted).

The small particles produced by sonic treatment can also be aggregated in the manner described above. An aqueous solution of 32 minute sonic treated material was made to aggregate by this method and was found to exhibit considerable stream birefringence although the original material had shown very little. The viscosity of solutions of this aggregated material was measured and it was found that the specific viscosity was twice that observed for the unaggregated sonic treated virus at the same concentrations. An electron micrograph of the aggregated material is shown in Fig. 4. There are many more curved particles in these samples than are usually observed

in normal samples of tobacco mosaic virus. The size distribution of the aggregated 32 minute sonic treated particles is shown in the unshaded area of Text-fig. 5. The size distribution is much broader than that for the unaggregated 32 minute sonic treated material shown in Text-fig. 2 and in the shaded area of Text-fig. 5. The biological activity of the aggregated sonic treated material was essentially the same as that of the unaggregated sonic treated material (3251 lesions for the former and 3169 for the latter).

The particles aggregate end-to-end more rapidly when the incubation temperature is increased. If a solution of sonic treated material in 0.1 M phosphate buffer at pH 7.0 is heated for 2 hours at its isoelectric point (pH 3.5 in buffer) at 60°C., and the pH is then brought back to 7.0, and is heated again at 60°C. for only 10 minutes the particles aggregate and the solution exhibits intense stream birefringence. Fig. 5 shows an electron micrograph, taken under low intensity, of 16 minute sonic treated material aggregated by this method. The points of junction of the particles are clearly visible.

DISCUSSION

Lauffer (16, 17) has shown that the stream birefringence and the high viscosity of solutions of purified tobacco mosaic virus are due to the elongated form of the particles. In the present work the decrease in intensity of stream birefringence and in viscosity with sonic treatment indicates a shortening of the particles. This conclusion is confirmed by direct observation in the electron microscope.

Sollner (18) found that vanadium pentoxide sols, known to contain highly asymmetric particles, show a marked decrease of stream birefringence on short sonic treatment. It is probable that the mechanism of breakage of these colloidal particles is similar to that operating in the breakage of the particles in samples of purified tobacco mosaic virus. There is no destruction of the vanadium pentoxide colloidal particles when cavitation, normally accompanying intense sound waves, is suppressed. This observation is in agreement with the observations of Stanley (2) that the biological activity of the tobacco mosaic virus samples is unchanged when cavitation is suppressed. Since cavitation is necessary for breakage of the particles and is accompanied by violent swirling, it is probable that the particles are broken by the strong macroscopic shearing stresses in the swirling set up in the liquid. The strong shearing forces accompanying cavitation might be expected to break elongated particles of colloidal dimensions in two since particles subject to Brownian movement but having superimposed random shearing forces should, from hydrodynamical considerations, show the greatest stress at the middle of the particles.

From the size distributions (Text-fig. 2) it appears that as a result of the sonic treatment the rod-like particles are broken in two, these halves broken

in two, and so on down to at least one-eighth the length of the 280 mμ rods. The various size distributions may be accounted for by the following approximate theory: Assume that any particle which is broken is broken in half and that every particle has an equal chance of being broken. (The latter assumption will be inaccurate for very short particles.) Assume also, for mathematical simplicity, that all the particles are initially of one length, L (nearly 280 mμ in our case). Then the following differential equations are simultaneously satisfied.

$$\frac{dN_L}{dt} = -kN_L \tag{1 a}$$

$$\frac{dN_{L/2}}{dt} = 2kN_L - kN_{L/2} \tag{1 b}$$

$$\frac{dN_{L/4}}{dt} = 2kN_{L/2} - kN_{L/4} \tag{1 c}$$

$$\frac{dN_{L/8}}{dt} = 2kN_{L/4} - kN_{L/8} \tag{1 d}$$

Equation 1 a expresses the rate of disappearance of particles which were initially all of the same length L, where k is the rate constant. Equation 1 b expresses the rate of appearance and disappearance of particles of length $L/2$. The first term on the right states that two particles are produced for every particle of length L which is broken. The second term expresses the disappearance of particles of length $L/2$ into smaller fragments. Equations 1 c and 1 d are the corresponding expressions for the rate of appearance and disappearance of particles of lengths $L/4$ and $L/8$ respectively.

Since we assume an homogeneous size L initially present, the above equations are accompanied by the boundary conditions: At time $t = 0$; $N_L = N_L(0)$, the number of particles of length L initially present, and $N_{L/2} = N_{L/4} = N_{L/8} = 0$.

Equations 1 a, 1 b, 1 c, and 1 d together with the boundary conditions can be shown to yield the solutions

$$N_L = N_L(0)e^{-kt}, \qquad N_{L/2} = N_L(0)e^{-kt}(2kt)$$

$$N_{L/4} = N_L(0)e^{-kt}\frac{(2kt)^2}{2!}, \qquad N_{L/8} = N_L(0)e^{-kt}\frac{(2kt)^3}{3!} \tag{2}$$

Thus, the number of particles of length L decreases exponentially with time; but the numbers of the smaller sizes go through maxima—the smaller the particles, the later the time at which the maximum occurs.

It is, of course, not possible to determine from the electron microscope the total number of particles in a sample but only the number per cent having certain lengths. Therefore, the number of particles of certain lengths at

time t given by Equations 2 must be divided by the total number of particles at time t. It can be shown mathematically that the total number of particles increases monotonically with time so that the form of the expressions for the number per cent of particles of given lengths as a function of time is that given by Equations 2. The size distributions given in Text-fig. 2 are seen to have the same functional relationship to time as those expressed by Equations 2. This similarity suggests that the theory is probably correct.

In the general case in which the particles are eventually broken into infinitely short pieces, the total number of particles of all possible sizes at a given time t is the summation of equations of the form of Equations 2 and is equal to $N_L(0)e^{kt}$, so that the number per cent of particles of length L at a given time is given by $100e^{-2kt}$; i.e., the number per cent of particles of lenght L decreases at twice the rate at which the total number of particles of this size is decreasing. It is unlikely, however, that the particles are broken much below one-eighth the size of the 280 mμ in length particles. Therefore, the actual total number of particles is somewhat lower than that given for the general case, and the rate constant for the decrease in number per cent of particles of length L is greater than k but less than $2k$, where k is the rate constant for the decrease in total number of particles of length L.

Since the biological activity decreased exponentially with time of treatment and since the number of particles 280 mμ in length decreased exponentially with time while the numbers of other size particles go through maxima, it is highly likely that *only the particles of length 280 mμ are the biologically active units*. The theory is substantiated by the fact that the rate constant calculated from Text-fig. 2 for the decrease in number per cent of particles 280 mμ in length is about 0.19 min.$^{-1}$, which is greater than the value for the rate constant for the decrease in biological activity ($k = 0.13$ min.$^{-1}$) but less than twice its value, $2k$. A more detailed correlation would require an elaborate numerical analysis.

The results of these experiments showing that only particles 280 mμ in length are biologically active is in complete agreement with the findings of Stanley and his coworkers (19), but is at variance with the suggestion of Bawden (20) that the 280 by 15 mμ rods are aggregates and that the primary virus particle is much smaller and not greatly elongated.

In the discussion above it is shown that the decrease in biological activity with sonic treatment is due to, or at least associated with, the mechanical destruction of the virus particles 280 mμ in length. The destructive effects of sonic treatment on certain proteins such as egg albumin have been attributed to the action of hydrogen peroxide (21), which is known to be produced in minute traces in water when cavitation is present (22). Sollner (23), however, in referring to experiments on the destruction of proteins by sonic treatment, states that "in many cases it seems likely that there is a kind of surface de-

naturation." As shown in the earlier part of this paper, the physicochemical evidence indicates that the tobacco mosaic virus was not denatured by the sonic treatment.

The results of studies of the effects of sound waves on other viruses are quite varied. Scherp and Chambers (24) have found that the pathogenicity of poliomyelitis, human influenza, and swine influenza viruses is not affected by sonic treatment; but Rivers, Smadel, and Chambers (25) found that the activity of elementary bodies of vaccinia is decreased by sonic treatment. The latter workers attributed the decrease of activity to the chemical effects of the hydrogen peroxide produced. Krueger, Brown, and Scribner (26) found that the biological activity of bacteriophage decreased on sonic treatment. More recently, Anderson (27) has observed, in the electron microscope, that numerous ghosts are found in samples of sonic treated bacteriophage. Probably in the case of bacteriophage the elaborate structure of the particles, as revealed in the electron microscope, is mechanically weak, while in the case of tobacco mosaic virus, the ease of mechanical destruction is probably associated with the highly elongated form of the particles.

The nature of the forces operating on the ends of the particles to bring about aggregation in samples of tobacco mosaic virus is, at the present time, not understood. The spaces, some as great as 30 mμ, at the junctions of the particles as seen in Fig. 5 suggest that the forces may act over distances much greater than do ordinary molecular forces. Examination with an electron microscope possessing a resolution higher than that of the instrument used in these studies may, however, reveal the presence of connecting links. The presence of numerous curved particles of the aggregated 32 minute sonic treated material shown in Fig. 4 suggests that there may be free rotation about the bonds connecting the particles.

The biological activity of normal virus samples decreases when the particles are aggregated because fewer active units are available to infect the susceptible points on the surface of the leaves. Text-fig. 4 shows that rapid stirring tends to break the aggregates into the monomeric units 280 mμ in length. Evidently the junctions of the virus units in the aggregates are mechanically weak points. The stirring is accompanied by an almost complete restoration of the activity of that for the normal virus sample.

The fact that the biological activity of the 32 minute sonic treated material did not change when the particles were aggregated indicates that it is not possible by this aggregation method to regenerate active particles by polymerization of their fragments, either because the proper type of bond is not formed between the fragments or because the fragments were brought together in a different sequence from that in which they occur in the normal particles or for both these reasons. It is highly unlikely that the fragments would aggregate in the proper sequence and be pointing in the correct direction with respect to one another. The activity of the 32 minute sonic treated sample

was not reduced by aggregation as it was when normal size particles were aggregated, probably because the number of normal size particles in the 32 minute sonic treated sample is relatively small. If random polymerization of the particles is taking place as indicated by the broad size distribution (Text-fig. 5), it is highly improbable that one normal size particle would aggregate with another of the same size.

SUMMARY

Centrifugally purified samples of tobacco mosaic virus were subjected to intense sound vibrations of 9,000 cycles per second for 0, 2, 8, 16, 32, and 64 minutes. The viscosity and stream birefringence of the samples decreased with time of sonic treatment, but no chemical changes were found. Electron micrographs of the samples show that the particles are broken perpendicular to their long axis. In the untreated sample 62 per cent of the particles are about 280 mμ in length. As sonic treatment continued, the number of particles of this length decreased exponentially with time, the number half this length increased and then decreased, and the number of quarter length particles subsequently increased and then decreased. The biological activity of the samples, as determined by the half leaf lesion method, decreased exponentially with time of sonic treatment with a rate constant given by $k = 0.13$ min.$^{-1}$. A correlation exists between the size distributions and biological activity and shows that only the particles of length 280 mμ are the biologically active units.

Tobacco mosaic virus particles can be made to aggregate end-to-end when the material is heated at its isoelectric point and reheated after being brought back to pH 7. Material which was not sonic treated and was made to aggregate showed reduced biological activity, but the activity was increased when the aggregated material was subjected to strong mechanical stirring. Material which was sonic treated for 32 minutes and which was made to aggregate showed the same biological activity as the material which was sonic treated but not aggregated.

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EXPLANATION OF PLATE 4

Electron micrographs of tobacco mosaic virus treated by various methods taken on RCA Console Model (type EMC-1) electron microscope. Magnification 22,000 and gold shadow cast.

FIG. 1. Centrifugally purified tobacco mosaic virus.

FIG. 2. Tobacco mosaic virus sonic treated for 16 minutes.

FIG. 3. Tobacco mosaic virus particles aggregated by the isoelectric heat treatment at a temperature of 37°C.

FIG. 4. Tobacco mosaic virus sonic treated for 32 minutes and aggregated by the isoelectric heat treatment at a temperature of 37°C.

FIG. 5. Tobacco mosaic virus sonic treated for 16 minutes and aggregated by the isoelectric heat treatment at a temperature of 60°C.
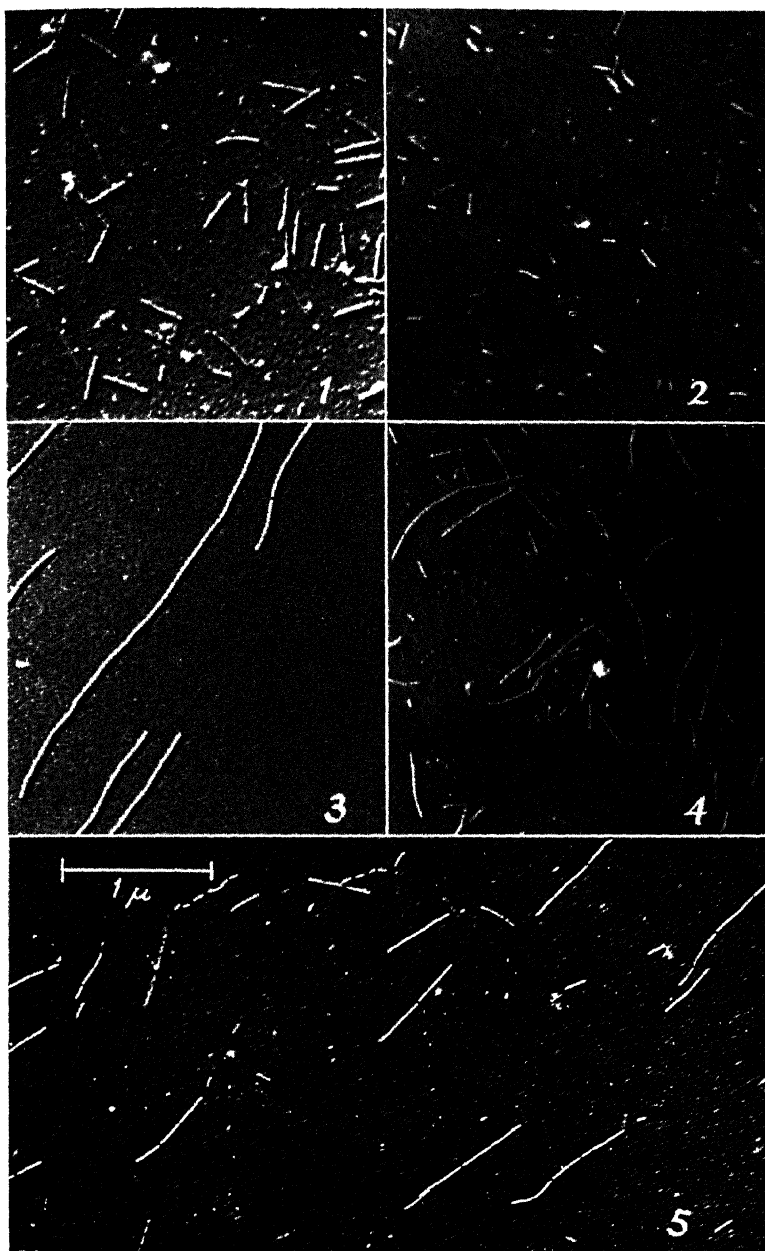
(Oster: Sonic treatment of tobacco mosaic virus)

STUDIES ON THE HEMOPHILUS GROUP OF ORGANISMS*

QUANTITATIVE ASPECTS OF GROWTH ON VARIOUS PORPHIN COMPOUNDS‡

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INTRODUCTION

In a previous paper (1) the function of some of the side chains of the porphin compounds was studied by utilizing the fact that certain strains of *Hemophilus* organisms required heme for growth. The present paper deals with the quantitative aspects of the growth of this group of organisms as affected by several iron porphins. Several substances have been found to potentiate the activity of the iron porphins and the mode of action of these substances has been discussed. The porphin requirement of one of these organisms during anaerobic growth has also been investigated.

Methods

1. *Organisms.*—All the hemophilus strains that have been studied have required coenzyme I. The Turner strain of *H. influenzae* was procured from Dr. C. L. Hoagland of the Rockefeller Institute. The various smooth types of *H. influenzae* were supplied by Dr. H. E. Alexander of Columbia University and by Dr. E. G. Stillman of the Rockefeller Institute. The *H. parainfluenzae* strains (including the strains Sibley, Fiddes, Langan, Leiter, Ogilvie, Paine, and Riley) were also procured from Dr. Alexander except for ATC No. 9796 which came from the American Type Culture Collection.

2. *Basal medium for growth of H. influenzae.*—The basal medium was similar to that described by Lwoff (2) and was prepared as follows: To make 1 liter, 20 gm. proteose-peptone (Difco), 6 gm. NaCl, 2 gm. KNO₃ were added to 1 liter of water and boiled vigorously for 10 minutes. The pH was adjusted to 7.4. One hundred mg. of sodium hydrosulfite were added with rapid stirring. The hot medium was filtered through paper, dispensed in convenient sized Erlenmeyer flasks, and autoclaved for 20 minutes at 15 pounds pressure. If a slightly turbid medium resulted due to insufficient preliminary boiling it was refiltered and reautoclaved. The hydrosulfite was added because, as we will show later, the organisms grew better on media that had been treated with a reducing agent. This hydrosulfite-treated medium was utilized in all the experiments reported.

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* This is the second of a series of studies on porphins and related compounds.

‡ The following nomenclature will be used for the purposes of this paper: porphin = porphyrin; heme = ferrous or ferric porphin.

3. *The coenzyme I* was prepared by the method of Williamson and Green (3). Tested spectrophotometrically by the extinction of its reduced form at 340 mμ it was found to be 12 per cent pure.

4. The *porphin compounds* were prepared as described in the previous publication (1).

5. The *porphin solutions* for addition to the medium were prepared as follows. The crystalline porphin was weighed out and dissolved in a solvent containing one part ethyl alcohol to one part 0.04 N aqueous KOH to make a stock solution containing 500 γ of the porphin per cc. From this stock solution dilutions were made containing 50, 5, and 0.5 γ per cc. in aqueous 0.02 N KOH. The *Hemophilus* organism could tolerate an alcohol concentration of 1.5 per cent, and occasionally no depression of growth was noted in 2 per cent alcohol. The maximum concentration of heme or porphin that could be added was limited by the alcohol content and was about 15 γ per cc. The alkali was used because the porphins are much more soluble in the form of their salts. The buffering capacity of the medium was such that the added alkali increased the alkalinity of the final culture by less than 0.1 pH unit.

The porphin solutions were not stable. It was found that the activity of iron porphin solutions, especially of iron protoporphin, rapidly decreased. Solutions of protoporphin and the other porphins did not deteriorate quite as rapidly but for precise work it was necessary to prepare the solutions fresh immediately before use, or to dilute them from the 500 γ per cc. stock solution. Even the stock solutions in 50 per cent alcohol were observed to deteriorate within 2 weeks.

6. *Procedure.*—To determine the effect of the porphins the following procedure was utilized. Four cc. quantities of the basal medium were pipetted into a series of test tubes. Coenzyme I was added, two to five times in excess of that required for optimum growth. In most of the experiments this consisted of 0.2 cc. of a solution containing 4 γ per cc. of the 12 per cent pure preparation of coenzyme I. Thus 0.019 γ of pure coenzyme I was present per cc. of medium. The solution of iron porphin or of the porphin to be tested was added in a series of appropriate dilutions to one set of tubes and as a standard iron protoporphin or protoporphin was added to another set. The volume was brought to 4.9 cc. with saline, and inoculated with 0.1 cc. of a culture prepared as described below. The cultures were incubated at 38°C. for 18 hours.

6 (*a*) The *inoculum* was prepared from a 6 hour culture inoculated from a 24 hour culture grown on the same medium; *i.e.*, the basal medium containing 0.019 γ per cc. of coenzyme I and 1 γ per cc. of iron protoporphin. After 6 hours' incubation the culture was turbid. It was diluted 1 to 50 with basal proteose-peptone medium, and 0.1 cc. was inoculated into each test tube. This method of preparing the inoculum had the following advantages: (1) it was reproducible; (2) it contained a constant small but effective number of organisms in the rapid stage of growth (the number of organisms in the inoculum averaged 1 $\times$ 10^7); (3) the heme content was very low, 0.002 γ of heme, not including that in the organisms themselves, being added in the 0.1 cc. inoculum; and (4) it gave a typical growth with known concentrations of heme and coenzyme under aerobic conditions.

Some strains of *Hemophilus* required more organisms in the inoculum and in such cases the 6 hour culture was sedimented, washed once with the basal medium by centrifugation, and then diluted one to ten. The heme added with the inoculum was still sufficiently low to be the limiting growth factor under aerobic conditions.

6 (*b*) *Evaluation of the growth of the organism.* The growth-supporting powers of the porphins in solution were compared by measuring the turbidity of the resulting cultures, and also by determining the amount of nitrate reduced to nitrite by the organisms.

A gross evaluation of the turbidity was made by observing the tubes visually. Turbidimetric readings were also made by measuring in a 1 cm. cell the intensity of transmitted light on the Beckman spectrophotometer at a wave length of 380 mμ.
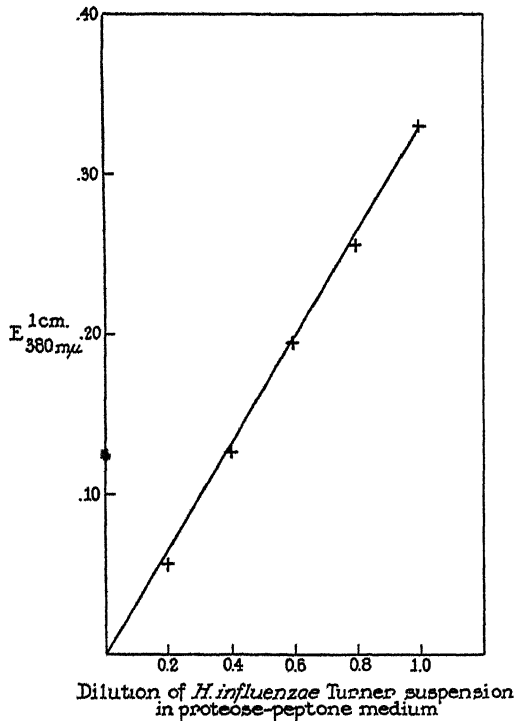

FIG. 1. The proportionality between the dilution of a suspension of *H. influenzae* Turner and the extinction at 380 mμ as measured in a Beckman spectrophotometer.

In order to remove the substances in the medium which absorb in this region, especially the porphin compounds added in varying amounts, the cultures were packed by centrifuging, the clear supernatant poured off, and the sedimented organisms resuspended in 6 cc. of the basal medium. The medium alone was used as the blank. In the case of experiments with ferrous ammonium sulfate, the colloidal ferric hydroxide was dissolved in acid before centrifuging. Turbidity measurement of tubes containing MnO_2 was feasible since this insoluble substance settled out rapidly and the culture could be decanted; in this case the blank was the medium plus MnO_2. The spectrophotometric readings made at 380 mμ were checked at 400 mμ. Readings which

were higher at 400 mμ than at 380 mμ indicated that porphins were vitiating the readings.

In order to discover whether turbidity thus measured was directly proportional to the number of organisms present, a heavy suspension of *H. influenzae* was diluted, roiled, and turbidity readings taken at various dilutions in the 1 cm. cell of the Beckman photoelectric spectrophotometer. Fig. 1 represents the results of such an experiment. It can be seen that there is a linear relation between the number of organisms, shown as the fraction of the original emulsion, and the extinction coefficient at 380 mμ.

The colorimetric method for the determination of the nitrite produced by the organisms is the modification by Hoagland (4) of the Shinn method (5).

7. *Procedure for anaerobic conditions.*—In a few experiments anaerobiosis was established by the use of a Brewer jar using hydrogen and a catalyst to remove the last traces of oxygen. In most of the anaerobic experiments oxygen was removed with a pyrogallol-alkali mixture. The procedure was as follows: A layer of pyrogallol crystals was placed on the bottom of a large desiccator with a beaker of 2 N alkali. The test tubes containing the organisms were placed in a rack in the desiccator. The desiccator was then evacuated and filled with purified nitrogen three times. At the last filling the nitrogen was held at a pressure of 10 cm. Hg. The alkali was then tipped onto the pyrogallol. Only a faint tinge of brown appeared, indicating that only traces of oxygen were present. A leak, if it occurred, could be readily detected by the darkening of the pyrogallol. The desiccator was placed in the incubator for the specified time. Before opening, it was brought to atmospheric pressure by filling with nitrogen.

EXPERIMENTS

I. Porphin Requirements of Hemophilus influenzae

Several types of *H. influenzae* have been recognized on the basis of morphology and certain metabolic characteristics, including their requirement of one or both of the growth factors, X and V. The X factor is a heat-stable compound associated with blood pigments. The V factor is heat-labile and is found in yeast and fresh animal and vegetable tissue. The typical *H. influenzae* requires both factors. Another group referred to as *H. parainfluenzae* requires only the V factor, and a third group requires only the X factor. It has been shown that the X factor can be replaced by iron protoporphin and that the V factor can be replaced by coenzyme I or coenzyme II (6, 7).

The use of a simple medium, that is, proteose-peptone, which we have found to be free of heme, and the availability of pure heme and relatively pure coenzyme, have made it possible to reclassify the rather heterogeneous group known as *H. parainfluenzae*. This was impossible in previous work where this group of organisms was isolated from throat washings in medium which included yeast extract as a source of coenzyme I.

(a) Hemophilus parainfluenzae.—

Only five of the eight strains purporting to belong to the *parainfluenzae* group on the basis of their ability to grow without the X factor could be grown

on our basal medium without the addition of iron protoporphin (Table I). The other three, Paine, Riley, and ATC No. 9796, could not be grown without iron protoporphin and therefore should be consigned to the *H. influenzae* group. It is probable that sufficient heme had been present in the yeast added to the medium used for isolation of these strains to permit them to grow.

The five strains of true *H. parainfluenzae* grew without heme. This was shown by passing them 15 times in serial transfer in proteose-peptone medium containing an excess of coenzyme I. The inoculum in each case was 0.2 cc. of

TABLE I

The Ability of Various Porphin Compounds to Support Growth of Several Strains of Hemophilus

Organism	Iron proto- porphin	Proto- porphin	Iron deutero- porphin	Iron meso- porphin	Iron hemato- porphin	Meso- porphin	Hemato- and deutero- porphin
			γ/cc. for +++ growth				
H. parainfluenzae Sibley Fiddes Langan Leiter Ogilvie	Not re- quired* (reduces NO₂)	Not re- quired* (reduces NO₂)	Not re- quired*	Not re- quired*			
H. influenzae (rough) Turner	0.01–0.10 (reduces NO₂)	0.01 (reduces NO₂)	0.25–0.50	0.01–0.05	>1.2	0.005–0.01 (more in- hibits)	No growth
H. influenzae (smooth) Types a,b,c,d,e,f	0.10–0.20 (reduces NO₂)	0.05–0.10 (reduces NO₂)	0.10–0.50	0.05–0.10	1.0–2.0	No growth	No growth
H. influenzae Paine Riley ATC No. 9796	1.0–2.0 (reduces NO₂)	0.1–0.2 (reduces NO₂)	No growth	No growth	No growth	No growth	No growth

* Except when inoculum is small.

the 5 cc. culture of the previous day, or approximately 2×10^8 organisms. Growth was excellent in all the tubes of the series, although it varied somewhat from one strain to the other.

If the size of the inoculum was decreased, that is to 0.2 cc. of a 1/50 dilution of a 6 hour culture (approximately 2×10^6 organisms), no growth occurred. However, if iron protoporphin or iron deuteroporphin was added to the medium, growth occurred, even with the smaller inoculum. The explanation for this phenomenon was suggested by further experiments. It was found that substances other than iron porphins had a similar effect in permitting growth of the small inoculum. Such substances included ferrous ammonium sulfate, sodium dithionite, catalase, and manganese dioxide (see below). These sub-

stances have one property in common: they destroy hydrogen peroxide either by reducing it (*i.e.* ferrous ammonium sulfate and sodium dithionite) or by catalyzing its decomposition (*i.e.* catalase and MnO_2). The destructive action of H_2O_2 on heme compounds is well known. It is therefore suggested that these substances made possible the growth of small inocula of *H. parainfluenzae* by decreasing the rate of destruction of the small amounts of heme being produced by the organisms. As these organisms continued to multiply they would develop a reducing environment and thus protect the heme that was being produced.

Attempts were made to train the strain *H. parainfluenzae* Sibley, to require heme by repeated passages on media containing heme. This was not accomplished.

(b) *Hemophilus influenzae.*—

A number of organisms of the *H. influenzae* group were studied. From their porphin requirements they may be divided for convenience into three subgroups (Table I).

1. H. influenzae Turner.—This organism was a rough strain which was carried for years by biweekly transfers on chocolate agar. It was better adapted to growth on proteose-peptone medium than any of the other organisms studied. Iron protoporphin, protoporphin, and the iron porphin compounds of meso- , hemato- , and deuteroporphin, all supported its growth. The organisms reduced nitrate to nitrite only in the presence of a vinyl-containing porphin; *i.e.*, protoporphin or iron protoporphin. The compounds, hematoporphin, deuteroporphin, and coproporphin not only did not support growth but actually inhibited the growth of the organism. A peculiarity of the Turner organism which differentiated it from the other *Hemophilus influenzae* organisms was that low concentrations of mesoporphin (0.01 to 0.05 γ per cc. of medium) supported its growth. Higher concentrations of mesoporphin however inhibited its growth. These phenomena were discussed in a previous paper (1). The quantitative aspects of the growth of this organism on various porphins is considered below.

2. H. influenzae Smooth Types a, b, c, d, e, and f.—The smooth types of the *H. influenzae* group have been classified on the basis of their antigenic properties (8). We examined representatives of the six known types and they had identical porphin requirements. They differed from the rough Turner strain only in the fact that mesoporphin did not support their growth at any concentration. This difference could not be attributed to the smoothness of the organism *per se*, since when two of the smooth strains, types c and d, were changed to rough by serial passages on medium containing homologous serum,[1] mesoporphin

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[1] The immune sera were kindly supplied by Dr. E. G. Stillman.

would still not support growth at any concentration. None of these smooth types grew as well on the proteose-peptone medium, nor from as small an inoculum as did the Turner organism. This possibly indicated slight differences in nutrient requirements among these organisms.

3. *H. influenzae Paine, Riley, and ATC No. 9796.*—As was stated above, there were three strains in the group of *H. parainfluenzae* organisms which were found to require iron protoporphin when grown on proteose-peptone medium. The porphin requirements of two of these strains, Paine and Riley, were studied. Unlike the other organisms in the *H. influenzae* group the vinyl-containing porphin compounds, protoporphin and iron protoporphin, were the only ones that supported their growth. Evidently, the vinyl groups of protoporphin were essential for some vital enzyme function in these two strains. With regard to the essential requirement of protoporphin or iron protoporphin, the Paine and Riley strains resemble the flagellated Strigomonas studied by the Lwoffs (9).

The data described above on the porphin requirements of the various strains of *Hemophilus* organisms have been summarized in Table I. The *H. parainfluenzae* group required no porphin unless the inoculum of organisms was small. The requirements of *H. influenzae* Turner were satisfied by a number of iron porphins, by protoporphin, and by low concentrations of mesoporphin. The smooth types differed from the Turner strain only in their inability to grow on mesoporphin. The Paine and Riley strains of *H. influenzae* grew only in the presence of protoporphin and iron protoporphin.

II. Quantitative Studies of H. influenzae Turner on Various Porphins

The growth-promoting porphins and their iron complexes differ not only in the concentration of porphin required for maximum growth of the organism but also in the amount of growth which is maximum for each compound. The compounds also differ in their toxicity to the *Hemophilus* organisms in high concentrations. The evaluation of the effectiveness of the porphins in promoting growth is complicated by their differences in solubility and in stability in solution. Besides this there are other factors, not at present subject to experimentation, such as the rate of penetration of the porphin into the organism, the rate of the incorporation of iron into it, the rate of utilization of the porphin to form a particular enzyme, and the rate of its destruction.

The ability of the various porphins to promote growth of *H. influenzae* Turner was compared. The organisms were grown on different concentrations of the compounds for 18 hours. The amount of growth was determined by measuring the absorption at 380 mμ in 1 cm. cells in the Beckman spectrophotometer. Fig. 2 illustrates one of these experiments. Under the experimental conditions, the extinction was directly proportional to the number of organisms present (see Fig. 1).

(a) The Porphins.—

Protoporphin was the most effective compound in supporting growth, readily surpassing iron protoporphin in this respect. Evidence was presented in the previous paper (1) showing that the organism inserts iron into protoporphin. Attempts were made to deplete the medium of iron in order to determine whether protoporphin was acting in any other capacity than as iron protoporphin for the support of growth. Although several methods were utilized, in-
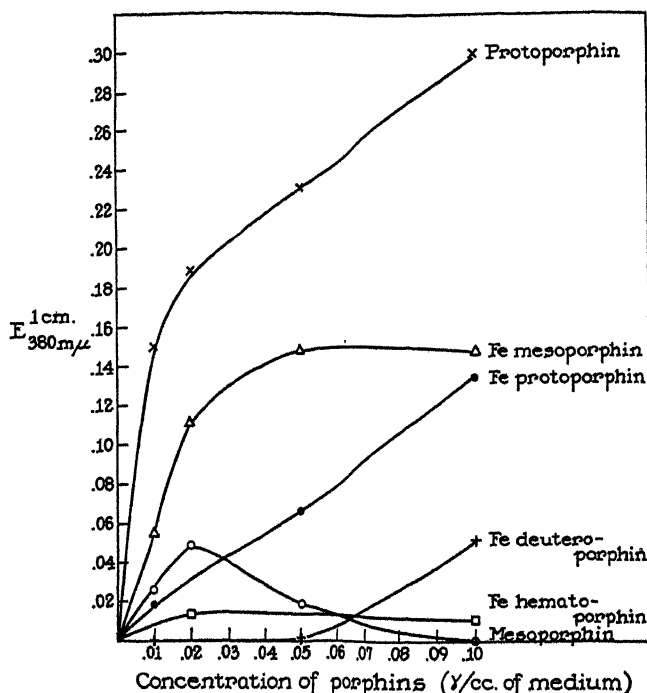
FIG. 2. The extinction turbidities of *H. influenzae* Turner grown for 18 hours on a proteose-peptone medium containing various concentrations of porphins.

cluding alkalinization of the medium, coprecipitation with calcium phosphate, dithizone and 8-hydroxyquinoline extraction, dipyridyl addition, and treatment with H_2S, the organisms grew in the presence of protoporphin as well as in the presence of iron protoporphin. Some of these media, for example the one treated with H_2S, were toxic and inhibited growth in the presence of protoporphin, as well as in the presence of iron protoporphin. Iron protoporphin could always be identified in the organisms grown on protoporphin. It is probable that the proteose-peptone medium clings to the iron too avidly to permit the removal of the traces which are sufficient for the synthesis of iron protoporphin.

Mesoporphin was the only non-iron porphin other than protoporphin which supported growth. As the quantity was increased, growth reached a maximum at a concentration of 0.02 γ per cc., then growth decreased and the compound became inhibitory above 0.1 γ per cc. Frequently, although not in this particular experiment, the slope of the curve at low concentrations was similar to that of protoporphin at the same concentrations. This suggested that the stability and the permeability of protoporphin and mesoporphin were of the same order.

(b) The Iron Porphins.—

Iron protoporphin was not as effective as protoporphin in supporting growth. This difference may be related to the relative ease of destruction of iron protoporphin as compared with protoporphin by H_2O_2. For example, a dilute solution of heme in 50 per cent alcohol was rapidly destroyed by 0.3 per cent H_2O_2 whereas a similar protoporphin solution remained unchanged. Iron protoporphin is known to have peroxidase activity. When protoporphin was mixed with iron protoporphin and the mixture treated with H_2O_2 both the iron protoporphin and the protoporphin were destroyed.

In suboptimal concentrations iron mesoporphin supported more growth than did iron protoporphin. However, when the compounds were supplied in excess, the maximum growth supported by the vinyl-containing porphyrins was always greater than the maximum growth supported by iron mesoporphin. This is not shown in Fig. 2 since maximal growth on heme was not attained at the concentration of 0.10 γ per cc. Iron deuteroporphin was less active than iron mesoporphin, and iron hematoporphin had only one-tenth the activity of iron protoporphin.

The differences in the behavior of the various iron porphins are probably not due to differences in solubility or stability. A more reasonable explanation is that these compounds function with relative degrees of effectiveness on the apoenzyme surfaces. The attainment of a much greater maximum growth with the vinyl-containing porphins suggests that although the vinyl groups are not essential for growth of the organism, they may be essential for the formation of some particular enzymes which aid in attaining maximum growth.

(c) The Potentiating Activity of Some Substances on the Growth of H. influenzae Turner.—

The toxic effect on microorganisms of hydrogen peroxide and of too high concentrations of oxygen has long been known (13). The growth of many organisms is improved by the addition of reducing substances to the media. In the case of the influenza bacillus the effectiveness of such substances seems to depend at least in part on their ability to protect heme from destruction.

A number of substances, when added to the medium containing iron porphins in suboptimal concentrations were observed to enhance the growth of *H.*

influenzae Turner. The protective effect of crystalline catalase and MnO_2 powder on the growth-promoting properties of the four iron porphins is shown in Fig. 3. The catalase was sterilized by filtration and added in a concentration of 0.1 γ per cc. of medium. This amount contained 0.001 γ of heme, a concentration which was too low to support growth or to improve growth notice-
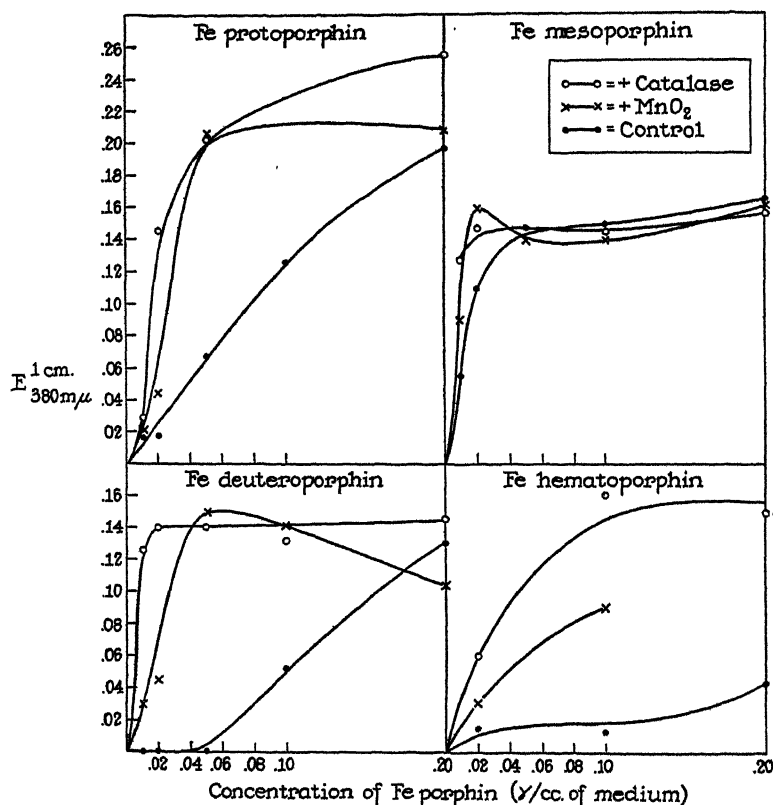
FIG. 3. The enhancement of growth of *H. influenzae* Turner on iron porphins by catalase and manganese dioxide.

ably. The action of catalase in improving growth on iron porphins was destroyed by boiling. The manganese dioxide was added to the medium in a concentration of 8 mg. per cc. before autoclaving. At this concentration the greater part of the MnO_2 did not dissolve and settled out rapidly to the bottom of the tube.

Fig. 3 shows the 18 hour growth represented in extinction units of turbidity on the ordinates and the concentration of iron porphins on the abscissa in γ per cc. It shows that at suboptimal concentrations of all four iron porphins growth

was increased by catalase and manganese dioxide. Catalase was superior to manganese dioxide in this effect. In contrast to the effect on the iron-containing porphins, catalase did not enhance the growth on protoporphin, and MnO_2 appeared to depress the growth on protoporphin. This depressant effect is still to be explained. The ability of catalase and MnO_2 to potentiate the growth-promoting power of the iron porphins may be most readily explained by the activity which they have in common of catalytically decomposing H_2O_2.

Several reducing substances were found to improve growth. Ferrous ammonium sulfate in a concentration of 10 γ per cc. improved growth in an iron protoporphin–containing medium and tended to raise it to the level of a protoporphin-containing medium. The growth on iron porphins was also improved by treating the medium before autoclaving with sodium dithionite at a concentration of 100 γ per cc. It is probable that these reducing substances destroyed peroxides which were either present in the medium or which developed during the growth of the organism. It appears that a reducing agent to be effective must not only reduce H_2O_2 in the medium but must also be able to reduce the H_2O_2 formed during the autoxidation of the reducing agent itself. This is perhaps the reason why ascorbic acid proved to be ineffective in protecting iron protoporphin. The addition of glucose or sodium bisulfite to the medium did not improve growth. Cysteine (0.04 to 0.2 mg. per cc. of medium) and thioglycollic acid (1.8 mg. per cc. of medium) were slightly toxic. The medium treated with H_2S prior to autoclaving was very toxic.

The inhibitory effect of H_2O_2 was determined directly by adding H_2O_2 in known concentrations to the medium. When the organisms were incubated for 18 hours with 0.05 γ of iron protoporphin per cc. of medium in the presence of H_2O_2, it was found that H_2O_2 in a concentration of 1.4×10^{-5} M in the medium was markedly inhibitory and that 1.4×10^{-6} M depressed growth by 25 to 50 per cent. If iron protoporphin and H_2O_2 were added to the proteose-peptone medium and incubated for an hour before the organisms were inoculated, then a marked inhibition of growth was observed at a concentration of 1.4×10^{-6} M H_2O_2. If the inoculum of organisms was increased tenfold growth was depressed by a concentration of 1.4×10^{-5} M H_2O_2, but not by a concentration of 1.4×10^{-6} M H_2O_2. No inhibitory effect of H_2O_2, in the highest concentration used (i.e. 1.4×10^{-5} M H_2O_2), was observed on the growth of the organism if protoporphin was used in place of iron protoporphin.

(d) Biological Assay of Porphins.—

The survey of the porphin requirements of the *Hemophilus* organisms (Table I) and the quantitative studies of growth as related to the concentration of the various porphins (Fig. 2) suggest that they may be adapted to a method for the assay of the porphins. Such a method would be ten to one hundred times more sensitive than the most sensitive physical methods. Single compounds,

such as protoporphin or iron protoporphin, could be distinguished from the other tetrapyrroles by the ability of these organisms to reduce nitrate in the presence of these compounds. The strains Paine and Riley could also be used for this differentiation since they grow only on protoporphin or iron protoporphin. Mesoporphin can be identified biologically by the fact that the Turner strain grows in the presence of low concentrations of this substance. The method is at present serviceable for the identification of certain porphins. Unfortunately it cannot be applied quantitatively directly to biological materials containing mixtures of porphins without preliminary separations which are tedious and in themselves are not particularly quantitative.

III. Iron Protoporphin Requirements of H. influenzae Turner under Anaerobic Conditions

Several authors have reported that under anaerobic conditions *H. influenzae* could grow in the absence of heme (10–12). When the V factor was supplied as potato water, it was found that such organisms grew well anaerobically without added heme. Few organisms could be grown anaerobically without added heme if the V factor was supplied as a vitamin B yeast concentrate. The above experiments do not rule out the possibility that heme might be required for anaerobic growth since it is probable that the potato water contained heme and that the yeast concentrate also contained traces of heme. In a reinvestigation of this problem, using *H. influenzae Turner*, this claim of anaerobic growth in the absence of heme was not confirmed.

Several considerations are of importance in these experiments. The organisms which require heme anaerobically, have a lower heme requirement under anaerobic than under aerobic conditions. (This is understandable if one recalls the rapid destruction of heme aerobically by peroxidative fission of the ring (14).) Furthermore, serial transfers are required in order to eliminate the traces of this factor in the initial inoculum. In this group of organisms one must consider, in addition, not only the traces of heme that might be present in the original media as impurities, but also traces that were possibly elaborated by organisms of the *Hemophilus* group themselves (*i.e.* traces of heme too low to support growth except under special conditions).

Table II summarizes an experiment dealing with the question of whether the Turner organism specifically requires heme when grown anaerobically. Anaerobiosis was effected by two reliable means (see methods above). The inoculum for the first experiment, No. 1, was 0.1 cc. of a 6 hour culture grown on 0.02 γ per cc. of heme. The inoculum for experiments 2 and 3 was 0.1 cc. of a culture containing 0.1 γ per cc. of heme from the previous experiment.

In order to prove that these inocula, grown anaerobically, were viable and would survive the time required for inoculation transfer under aerobic conditions, duplicate samples of the inoculum were tested for viability by incubating them aerobically at 38° in medium containing no heme; then after $3\frac{1}{2}$ hours

excess heme was added.   If the organisms grew out, one could conclude that the aerobic transfer of the inoculum from one anaerobic experiment to the next was not the cause for the absence of growth.

The table shows that in the first passage (No. 1) the organisms grew anaerobically without added heme.   In the second passage (No. 2) there was no growth unless heme was added.   Likewise in the third passage (No. 3) heme was required for anaerobic growth.   This experiment indicates that heme in small amounts is required for anaerobic growth.   The relatively large amount of heme needed aerobically is due in part at least to the instability of heme under aerobic conditions.

TABLE II

*The Necessity of Heme for Successive Passages of H. influenzae Turner under Anaerobic Conditions**

Anaerobic passage No.	Incubation time	Turbidity in media containing heme in a concentration of:		
		0 γ/cc.	0.01 γ/cc.	0.10 γ/cc.
	hrs.			
1	28	0.087 E	0.088 E	0.094 E
2	50	0.00	0.054	0.075
3	72	0.00	0.055	0.045

* All experiments were run in triplicate.   All tubes were evacuated and filled with N_2 before inoculation.   All tubes contained 4 cc. proteose-peptone + 0.3 cc. coenzyme I (0.028γ of pure compound), diluted with saline to final volume of 5 cc.   Controls, run without coenzyme, showed no growth as was to be expected if the cultures were not contaminated. During transfers the cultures were exposed aerobically for less than 20 minutes before they were returned to anaerobic conditions.   Turbidity was measured by determining extinction of suspension of organisms at 380 mμ and 1 cm. light path.

The turbidity extinction at a concentration of 0.1 γ of heme per cc. in the anaerobic experiment illustrated above was in the first passage 0.094, in the second passage 0.075, and in the third passage, 0.045.   Aerobically with a similar optimum concentration of heme the extinction varied between 0.120 and 0.220 in many experiments and there was of course no decline on serial passage.   It is obvious therefore that anaerobic growth is not only much poorer but that there is also a likelihood that the organisms would not have survived many more anaerobic passages.   At any rate the fact that heme is required by this organism in order to grow anaerobically has been demonstrated.   These experiments also show that some other factor or factors limit growth under anaerobic conditions.

DISCUSSION

The classification of the *Hemophilus* organisms into those requiring heme (*H. influenzae*) and those not requiring heme (*H. parainfluenzae*) suggests that a

sharp division between these two groups might have been brought about by a genic loss of some metabolic step necessary for the synthesis of heme.

Those organisms which can grow without added heme presumably make their own heme, the level of heme in a cell being dependent upon the rate of heme synthesis and the rate of its destruction. If the level of heme were relatively slight in some strains of *H. parainfluenzae* it might happen that these strains might appear to require heme for growth under certain experimental conditions and not under other conditions. Such strains have been found. When factors in the medium were present to diminish the rate of heme destruction these organisms were found to grow without added heme.

It appears from the above experiments that traces of peroxides are constantly being formed in culture media such as proteose-peptone under aerobic conditions. These peroxides are present in such minute amounts that they cannot be detected readily by direct chemical methods. Peroxides are known to destroy heme almost specifically. Agents or conditions which destroy the peroxides actually bring about a stimulation of growth by preventing the destruction of heme. Several seemingly unrelated phenomena are explained by the destructive action of peroxides on heme: (1) Reducing substances such as hydrosulfite, ferrous ammonium sulfate, etc., stimulate growth. (2) Agents which destroy hydrogen peroxide such as catalase and MnO_2 stimulate growth. (3) Small inocula of *H. parainfluenzae* of certain types do not grow unless supplied with heme, whereas large inocula of the same types grow readily in media devoid of added heme. (4) Much smaller amounts of heme are required for anaerobic growth than for aerobic growth of *H. influenzae* Turner.

SUMMARY

The porphin requirements of the *Hemophilus* organisms have been studied. Organisms of the *parainfluenzae* group show quantitative differences in their ability to synthesize heme. The ability of the *parainfluenzae* organisms to grow appears to depend on the rate with which they synthesize heme and in part at least on the properties of the medium to protect the heme from peroxidative breakdown.

Quantitative studies of the growth of *H. influenzae* Turner on various iron porphins have been made. Iron protoporphin gives greatest growth when supplied in excess, although iron mesoporphin appears more efficient at lower concentrations. Iron deutero- and iron hematoporphin are much less effective. This suggests that although the vinyl groups are not essential for growth of the Turner organism they may be required for some particular enzymes which aid in attaining maximum growth.

A number of substances potentiate the growth-promoting properties of iron porphins. These substances include reducing agents and agents which destroy H_2O_2.

H. influenzae Turner appears to require heme for anaerobic as well as aerobic growth.   The possibility of an essential heme enzyme functioning under anaerobic conditions must therefore be considered.

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BACTERIOPHAGE FORMATION WITHOUT
BACTERIAL GROWTH

I. Formation of Staphylococcus Phage in the Presence of Bacteria Inhibited by Penicillin

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The bacteria-bacteriophage system lends itself to a study of virus-host relationships better than other systems. The experimental conditions may be carefully controlled and experiments performed rapidly. In addition, the results represent a statistically significant number of individual host-virus systems.

The first step in such an analysis would be the separation of phage production from the growth of the host cells. Krueger and Fong (1), Gratia (2), Northrop (3), and Ellis and Spizien (4) have reported small increases in phage without bacterial growth. The possibility of the multiplication of a small number of cells, however, was not ruled out. Since a small increase in cells can give rise to fairly large increases in phage, it is imperative to measure the growth of the bacteria at frequent intervals by an accurate method.

Using igepol, a detergent, various pH, lysozyme, and ultraviolet irradiation, Northrop found that as soon as the growth of *B. megatherium* or *Staphylococcus muscae* was inhibited, the growth of the corresponding phage was prevented (5).

Evidence will be presented in this paper to show that bacteriophage can increase in the presence of bacteria whose growth has been inhibited by penicillin. In the second paper of this series experiments will be reported which indicate that penicillin prevents the utilization of a certain substance by bacteria which is essential for phage production (6). The third paper shows that adenosine-triphosphate is important in the formation of phage (7).

RESULTS

The addition of more than 10 γ of penicillin per ml. completely prevents the multiplication of *Staphylococcus muscae* (Table I). The addition of penicillin even in very high concentrations does not inhibit phage production (Table II). Fig. 1 gives the results of an experiment in which the cell concentration and phage concentration were determined at intervals in a culture containing 20 γ of penicillin per ml. The cell count was determined by a photoelectric color-imeter. The method is accurate to ± 2 per cent (Table IV). The curve in

Fig. 1 shows that there were no significant changes in the cell concentration and that a change in concentration of ± 3 per cent could have been detected.   The plaque count on the other hand rose from 10^4 to 3×10^8.   This experiment was repeated many times with the same result.

Effect of Initial Cell Concentration on Phage Production.—Under normal conditions more phage is produced by the inoculation of a system containing less than the maximum number of cells capable of growing in the media, than by the

TABLE I

Effect of Penicillin Concentration on Bacterial Growth

Each tube contained 5.0 ml. of broth and 0.5 ml. of Locke's solution with various penicillin concentrations.   Growth measured at 4 hours.   Two readings taken for each determination.

Tube No.	Penicillin concentration	Initial cell count	Final cell count
	$\gamma/ml.$		
1	2	$1.0–1.1 \times 10^8$	$2.1–2.1 \times 10^8$
2	5	$0.9 \times 10^7–1.1 \times 10^8$	$1.5–1.6 \times 10^8$
3	10	$1.0–1.0 \times 10^8$	$1.0–1.1 \times 10^8$
4	20	$1.0–1.1 \times 10^8$	$1.0–1.0 \times 10^8$

TABLE II

Phage Formation at Various Penicillin Concentrations

Each tube contained 5.6 ml. of broth, 0.7 ml. of broth containing 2.1×10^5 phage plaque counts per ml., and 0.7 ml. of broth containing various penicillin concentrations.   Initial bacterial count was 3.0×10^8 cells per ml.   Samples taken at 8 hours when all tubes were completely lysed.

Sample	Penicillin concentration	Initial phage plaque counts per ml.	Final phage plaque counts per ml.
	γ per ml.		
1	20	2.1×10^5	9.4×10^8
2	100	2.1×10^5	1×10^9
3	500	2.1×10^5	8.1×10^7

inoculation of a culture which already contains the maximum number of cells (8).   In the presence of penicillin, however, the opposite result is obtained. More phage is produced in the concentrated suspension than in the dilute cell suspension (Table III).   The addition of penicillin therefore increases phage production in the concentrated suspension but decreases it in the dilute suspension.   The increase in the concentrated suspension is due to the fact that the penicillin prevents the cells from using up a compound which is essential for phage production.   This observation will be discussed in the following paper.

    The decrease in phage production in dilute suspension is probably due to the

fact that the normal culture grows rapidly so that many more bacterial cells are present at the time of lysis than in the penicillin culture.
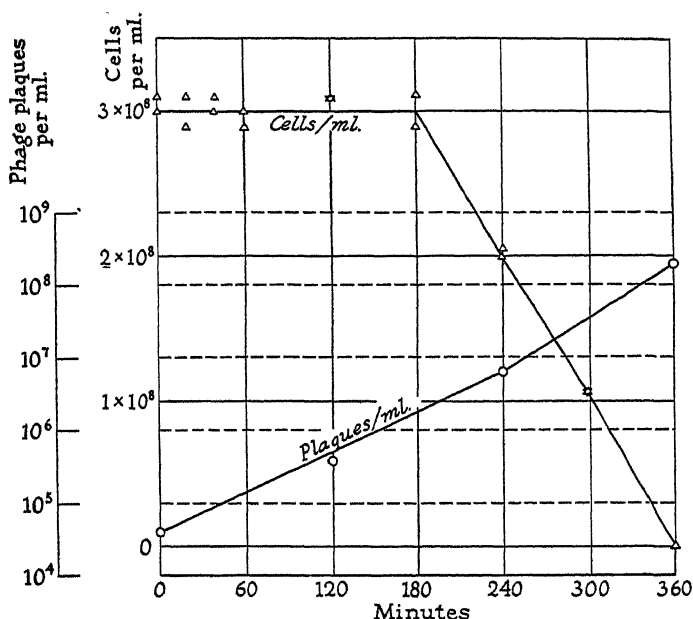
FIG. 1. Lysis of bacteria with formation of bacteriophage in the presence of penicillin. Sample contained 5.6 ml. of broth, 0.7 ml. of broth containing 140γ of penicillin, and 0.7 ml. of phage solution. Two readings taken for each cell determination.

TABLE III

Effect of Initial Cell Concentration on Phage Produced from Normal and Penicillin-Treated Bacteria

All tubes contained 5.6 ml. of broth, 0.7 ml. of phage solution, and 0.7 ml. of 0.85 per cent NaCl with and without penicillin. Samples taken at end of 6 hours.

Tube No.	Penicillin concentration	Initial cell count	Maximum cell count	Initial phage plaque count per ml.	Final phage plaque count per ml.
	$\gamma/ml.$				
1	—	8×10^8	1×10^9	4×10^4	3×10^6
2	—	5×10^7	1×10^8	4×10^4	3×10^8
3	20	8×10^8	8×10^8	4×10^4	4.8×10^9
4	20	5×10^7	5×10^7	4×10^4	2×10^6

From Table III it can also be seen that normal bacteria produce, under our method of assay, 3 plaques per bacterial cell, while the penicillin-treated organisms produce 1 plaque per cell. The difference between these two figures
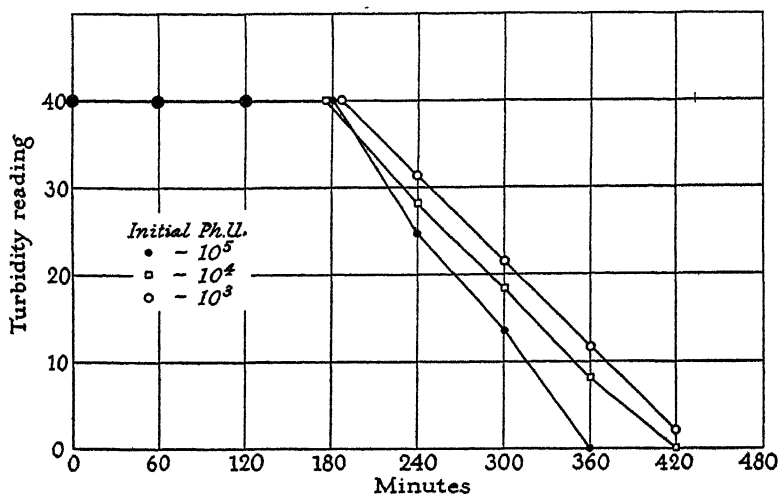
FIG. 2. Bacterial lysis at various initial phage concentrations. Each tube contained 5.6 ml. of broth, 0.7 ml. of broth containing 140γ of penicillin, and 0.7 ml. of phage solution.
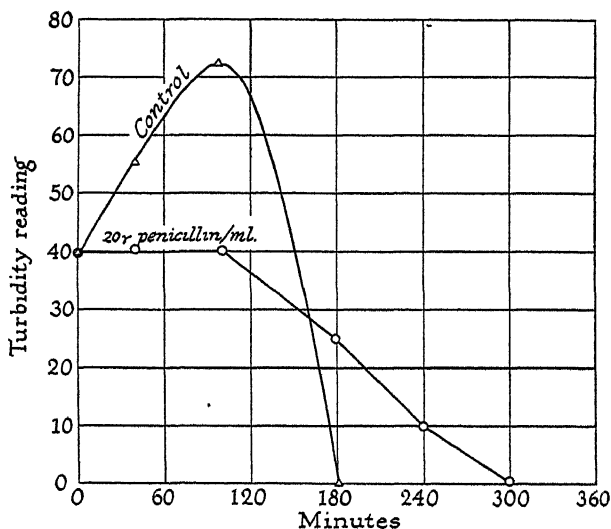
FIG. 3. Comparison of lysis of normal bacteria and penicillin-treated bacteria. Each tube contained 5.6 ml. of broth + 0.7 ml. of broth with or without penicillin and 0.7 ml. of broth containing 10^5 phage units per ml. Both samples showed approximately 10^9 phage units per ml. at complete lysis.

could conceivably be due to the fact that the penicillin is continually killing the cells and that there are really fewer viable cells in the penicillin culture than can

be determined by the turbidity method, which only measures total bacteria and does not distinguish between living and dead cells. This effect of penicillin on bacteria is well known.

It could be argued that in the presence of penicillin there is a continual slow multiplication of bacteria which is too low a concentration to be detected. This does not seem likely, since there does not appear to be any inhibition of phage formation in penicillin concentrations ranging from 20 to 100 γ (Table II). If the phage formation was due to undetected growth, one would expect that there would be a difference in phage formation under such conditions. The addition of 500 γ of penicillin results in less phage formation than adding 100 γ of penicillin. This is probably due to the fact that such high concentrations of penicillin kill the bacteria.

It should also be noted that not only is cellular multiplication inhibited, but that the cells do not grow at all, for any increase in the size of the bacteria could be detected with the colorimeter.

Effect of Phage Concentration on Lysis Time.—The rate of lysis of the penicillin-treated bacteria depends on the concentration of phage (Fig. 2). A 100-fold dilution of phage results in approximately a 60 minute increase in lysis time. This result agrees quite well with the observation of Krueger and Northrop (9) for normal bacteria.

The rate of lysis is slower in the presence of penicillin than in the normal culture (Fig. 3). The reason for this phenomenon is not known at the present time.

DISCUSSION

Penicillin prevents the multiplication of the *Staphylococcus muscae* but does not prevent the formation of the staphylococcus phage. This finding is important as it is the first step in an analysis of phage production. In systems where phage formation depends on bacterial growth a biochemical analysis is practically impossible, since all observations would be greatly complicated by variations in the growth of the host.

Cohen and Anderson (10) have reported that *E. coli* stop multiplying as soon as they are infected with T$_2$ phage. This observation is in agreement with the results presented in this paper that phage can be formed in the presence of non-multiplying bacteria. It should be pointed out, however, that inhibition of multiplication by the T$_2$ phage cannot be a universal bacteria-bacteriophage effect, for strains of *megatherium* are known which produce phage but continue to multiply (11).

SUMMARY

Bacteriophage will increase 100,000 times in *Staphylococcus muscae* cultures whose multiplication has been completely inhibited by penicillin.

I am indebted to Dr. John H. Northrop for advice during these experiments. I also wish to thank Dr. Richard E. Shope for a culture of the bacteria and virus used in this work.

Experimental Methods

Preparation of Standard Suspensions of Staphylococcus muscae.—18 to 24 hour bacterial cultures grown on veal infusion agar slants were washed off with sterile broth and incubated 2 to 3 hours at 37° unless otherwise stated.

Bacterial Concentration.—Bacterial concentrations were determined in a Klett-Summerson photoelectric colorimeter using 1.5 × 12 cm. sterile test tubes containing
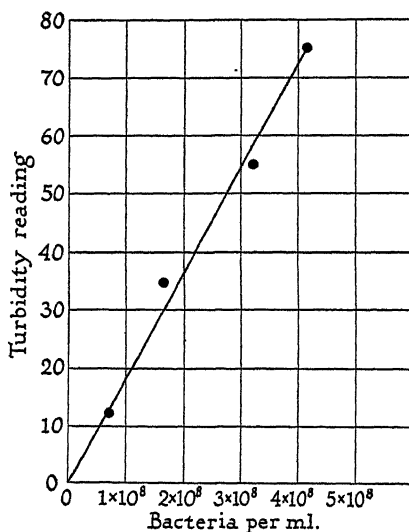
FIG. 4. Standard curve for determining bacterial concentrations.

5.0 cc. of the solution. The colorimeter was set for the reading by using 5.0 cc. of nutrient broth as the standard and reading against filter No. 54. Unknown values were read off a standard curve (Fig. 4). The curve was plotted from turbidity readings of standard suspensions in which the cell concentration was determined by microscopic count and checked by colony counts. Table IV shows five turbidity readings on the same sample. It can be seen that the accuracy is about ± 2 per cent for determining cell growth by this method.

Phage Assay.—Phage was determined by plaque counts according to Gratia (11). All dilutions were 1/10 and were made in broth. The final dilution prior to plating consisted of 3.5 ml. broth + 0.5 ml. phage sample + 1.0 cc. of 2.5 per cent agar. This mixture contained about 3 × 10^8 cells per ml. One ml. of the total mixture was pipetted into nutrient agar dishes and incubated about 18 hours at room temperature before counting the plaques.

Phage was also determined by the dilution method. In this method the pipette was rinsed in every dilution five times. All dilutions were 1/10 and made in broth.

The concentration of phage as determined by this method is expressed in phage units (Ph.U.). One phage unit, as determined by this method, is the smallest amount of phage solution that will cause lysis of 5 ml. of standard bacterial suspension containing 5×10^7 cells per ml. at $37°$ in 24 hours. The phage unit is the reciprocal of the maximum dilution which will cause lysis; *i.e.*, if 1×10^{-4}/ml. causes lysis [Ph.U.] /ml. $= 10^4$.

Preparation of Veal Infusion Broth.—To 500 gm. of ground veal is added 1000 ml. of H_2O and infused overnight in the ice box. The meat was strained next morning through fine cheese cloth and the filtrate made up to 1000 ml. with H_2O and heated in the steamer. Ten gm. of peptone and 5 gm. of NaCl was then added to every

TABLE IV

Accuracy of Turbidity Reading

Tube contained 5.0 ml. broth. Five separate readings taken, each time removing the tube from the colorimeter.

Reading	Value	Cells per ml. from Fig. 4
1	52	3.7×10^8
2	50	3.5×10^8
3	52	3.7×10^8
4	51	3.6×10^8
5	51.5	3.7×10^8
		3.64 ± 0.07*

* Average error of a single observation.

1000 ml. of broth. The pH was adjusted to 7.6 with N NaOH and the solution filtered through fine filter paper and autoclaved.

Penicillin.—Usually 20α of penicillin was added per ml. of reaction mixture to prevent multiplication. Several different samples of penicillin were used although most of the work was carried out with crystalline penicillin G sodium Squibb. The penicillin was incubated 90 minutes with the bacterial suspension before the addition of phage in order to ensure complete inhibition of bacterial growth. Ten α of penicillin per ml. gave complete inhibition of bacterial growth (Table I).

Phage.—All the experiments during this work were carried out with the staphylococcus phage described by Shope (12).

Reaction Mixtures.—All reaction mixtures were carried out in 2.0×15 cm. sterile test tubes, and were shaken rapidly without causing foam to appear. All experiments were carried out at $37°C$.

Addendum.—After this paper was accepted for publication, Dr. A. P. Krueger informed us that his group at the University of California had independently observed that *Staphylococcus aureus* phage would multiply in the presence of bacteria under conditions where penicillin prevented any demonstrable multiplication of bacteria.

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BACTERIOPHAGE FORMATION WITHOUT BACTERIAL GROWTH

II. The Effect of Niacin and Yeast Extract on Phage Formation and Bacterial Growth in the Presence of Penicillin

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In the preceding paper it was found that phage increases in the presence of bacteria whose multiplication was completely inhibited by penicillin. It was also reported that in concentrated bacterial suspensions there was more phage formed in the presence of penicillin than in the absence of the inhibitor. This observation suggested that in the presence of penicillin the bacteria did not utilize a substance which was essential for phage formation.

In this paper it will be shown that bacteria do not use niacin in the presence of penicillin and that niacin is essential for phage production. Experiments will also be presented which show that one or more factors are necessary for phage production besides those that are necessary for bacterial growth.

RESULTS

In broth diluted 1/17 with Locke's solution, the addition of penicillin to bacterial cultures containing 2.5 to 3.5×10^8 cells per ml. permits a good increase in phage. Very little phage is formed without the addition of penicillin (Table I). In bacterial suspensions containing less than 1.5×10^8 cells per ml., there is a large increase in phage without the addition of penicillin (Table II). These experiments suggested that penicillin by preventing the multiplication of bacteria allowed one or more substances to be used for phage formation that were normally utilized by the bacteria. In the presence of a low concentration of cells more of the substance can be utilized in phage formation.

As both niacin and thiamine were utilized for bacterial growth, each of these compounds was added to the dilute broth media containing 2.5×10^8 cells per ml. The addition of niacin alone without the addition of penicillin permitted the formation of phage with no detectable effect on bacterial growth (Fig. 1). Thiamine, pantothentic acid, biotin, pyridoxamine, and riboflavin were without effect on phage formation in the above system.

Subsequent experiments revealed that one or more substances were necessary for phage production besides niacin.

Bacteria were allowed to reach their maximum growth in the dilute broth system and then niacin with or without penicillin was added followed by the addition of phage. There was no increase in phage under these conditions. The addition of more ordinary nutrient broth permitted the formation of phage (Table III). This result suggested that the bacteria had used up one or more substances besides niacin normally present in broth which were essential for phage formation.

TABLE I

Phage Formation in Dilute Broth in the Presence of Concentrated Bacterial Suspensions with or without Penicillin

Each tube contained 5.0 ml. Locke's solution and 0.4 ml. broth and 1 ml. phage diluted in Locke's solution. Bacteria were centrifuged from broth culture, washed once with 5.0 ml. Locke's solution, and then resuspended in Locke's solution. To one tube was added 0.6 ml. of Locke's solution and to the other tube 0.6 ml. of Locke's solution containing 140 γ of penicillin. Total volume 7.0 ml. Sample was taken at the end of 8 hours when tube with penicillin showed complete lysis and tube without penicillin showed no lysis.

Tube No.	Additions	Initial cell count	Maximum cell count	Initial plaque counts	Final plaque counts
				per ml.	*per ml.*
1	None	2.5×10^8	4.3×10^8	4.6×10^5	1.1×10^6
2	20 γ penicillin/ml.	2.5×10^8	2.5×10^8	4.6×10^5	3×10^8

TABLE II

Phage Formation in the Presence of a Low Bacterial Concentration in Dilute Broth

Each tube contained 5.9 ml. of Locke's solution, 0.4 ml. of broth, and 0.7 ml. of phage solution diluted in Locke's solution. Bacteria were centrifuged, washed once with saline, and suspended in the 5.9 ml. of Locke's solution. Samples taken at end of 7 hours when both tubes were completely lysed.

Tube No.	Addition	Initial cell count	Maximum cell count	Initial plaque counts	Final plaque counts
				per ml.	*per ml.*
1	None	9×10^7	1.8×10^8	1.1×10^5	3×10^8
2	20 γ penicillin/ml.	9×10^7	9×10^7	1.1×10^5	4.8×10^7

It was thought of interest to determine whether the utilization of the unknown substance or substances by bacteria was prevented by penicillin. Two tubes were set up containing 1×10^9 cells per ml. in 1.5 ml. of broth. To one tube was added 50 γ of penicillin per ml. The two tubes were then incubated 5 hours at 37° and centrifuged. The effect of the supernatant fluid on phage production was studied by adding it to the synthetic medium of Fildes which does not suffice for phage production. From Table IV it can be seen that the broth in which the bacteria had been suspended no longer caused phage formation when added to the synthetic medium, whether penicillin was present or
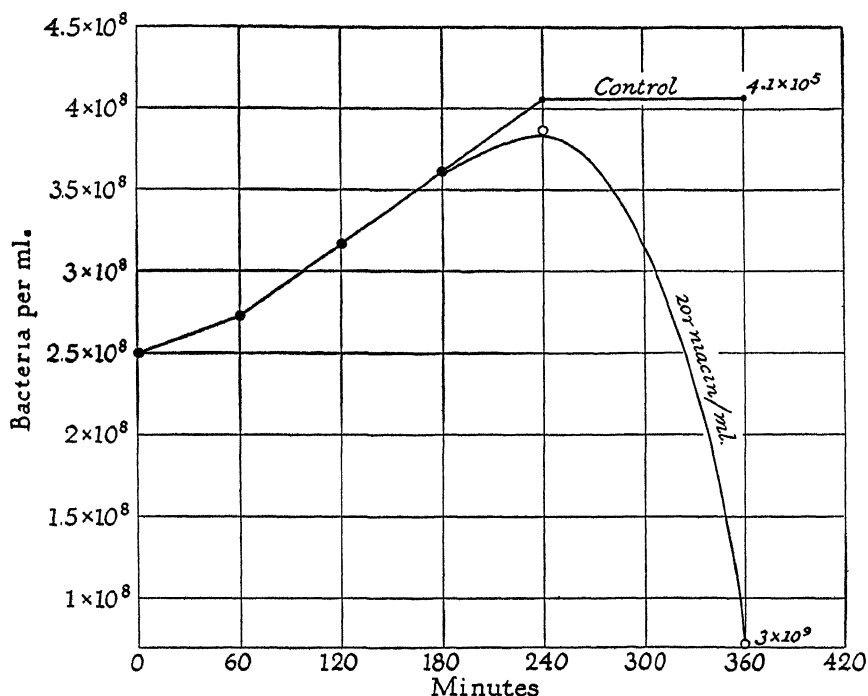
FIG. 1. Effect of niacin on phage formation in a fairly concentrated bacterial suspension. Each tube contained 5.9 ml. of Locke's solution, 0.4 ml. of broth, and 0.7 ml. of phage solution diluted in Locke's solution. Bacteria centrifuged, washed once with saline, and suspended in the 5.9 ml. of Locke's solution. Bacteria incubated 30 minutes before addition of phage and niacin. Initial plaque counts per ml. were 3.7×10^4. Figures at 360 minutes represent final phage plaque count per ml.

TABLE III

*Effect of Niacin and Broth on Phage Production after a 5 Hour Multiplying
Period of a Bacterial Culture*

Bacteria were washed from one agar slant with 15 ml. of nutrient broth and incubated for 2 hours at 37°. The suspension was then centrifuged, and the cells washed once with 15 ml. of saline and then resuspended in 20 ml. of a 1/13 dilution of broth in Locke's solution. 5.4 ml. of this suspension was put in 3 tubes. The initial cell count was 2.9×10^8 which rose to 4.2×10^8 after 5 hours' incubation at 37°. At this time 1.0 ml. of phage diluted 1×10^4 in Locke's solution, penicillin, and niacin was added as shown in Table III. Tubes shaken at 37°. After 8 hours cell count and phage count determined. No lysis in tubes 1 and 2. Complete lysis in tube 3.

Tube No.	Niacin	Peni-cillin	Locke's	Broth	Cell count		Plaques	
					Initial	Final	Initial	Final
	$\gamma/ml.$	$\gamma/ml.$	*ml.*	*ml.*			*per ml.*	*per ml.*
1	20	—	2.0	—	4.2×10^8	4.2×10^8	1.7×10^5	5.5×10^4
2	20	20	2.0	—	4.2×10^8	4.2×10^8	1.7×10^5	3.1×10^4
3	—	—	—	2.0	4.2×10^8	6×10^8	1.7×10^5	2×10^8

not. Evidently, the cells use one or more substances in the broth necessary for phage formation even in the presence of penicillin. This experiment explains the result in Table V. In this experiment it was found that bacterial suspensions over 4.0×10^8 cells per ml. show less phage formation than one would expect from Table I. From the results in Table IV it is obvious that by increasing the bacterial concentration less of the unknown factor or factors are available for phage production.

TABLE IV

Effect of Penicillin on the Utilization of the Unknown Phage Factors by Bacteria

Two tubes, A and B, contained 1.5 ml. broth plus 1×10^9 cells per ml. Tube B received 50 γ of penicillin per ml. Tubes then incubated 5 hours at 37° and centrifuged. 1.0 ml. of the supernatant fluid from samples A and B was respectively added to 2 tubes each containing 8.0 ml. of the synthetic medium and having 1.5×10^8 cells per ml. All tubes then incubated. Phage assay at end of 8 hours. Tube 1 completely lysed at this time. Tubes 2 and 3 were not lysed.

Tube No.	Additions	Initial plaque counts	Final plaque counts
		per ml.	*per ml.*
1	1.0 ml. normal broth	2.1×10^5	1.2×10^9
2	1.0 ml. from tube A	2.1×10^5	3.1×10^4
3	1.0 ml. from tube B	2.1×10^5	1.2×10^4

TABLE V

Effect of Increasing Bacterial Concentrations on Phage Production in Dilute Broth in the Presence of Penicillin

Each tube contained 5.2 ml. of Locke's solution, 0.4 ml. of broth, 0.7 ml. of Locke's solution containing 350 γ of penicillin, and 0.7 ml. of phage diluted in Locke's solution. Bacteria were centrifuged, washed once with saline, and suspended in the 5.2 ml. of Locke's solution. Phage assay taken at end of 8 hours.

Tube No.	Cell count per ml.	Initial phage plaque counts	Final phage plaque counts
		per ml.	*per ml.*
1	1.0×10^8	6.6×10^4	1.7×10^7
2	2.5×10^8	6.6×10^4	1×10^9
3	4.5×10^8	6.6×10^4	1.1×10^7
4	7.0×10^8	6.6×10^4	2.2×10^5

That one or more compounds besides niacin were necessary for phage formation was also indicated by experiments in which phage formation was studied in the synthetic medium of Fildes[1] (2). This medium permitted good bacterial

[1] This medium contains the following amino acids; alanine, valine, leucine, cystine, glycine, proline, oxyproline, aspartic acid, glutamic acid, methionine, phenylalanine, tyrosine, tryptophane, arginine, histidine, and lysine; also glucose, ferrous ammonium sulfate, niacin, thiamin, phosphate, sodium nitrate, and magnesium.

growth but did not form phage with or without penicillin unless broth or yeast extract was added (Fig. 2). A solution containing biotin, guanine, adenine, uracil, xanthine, and thymine, β-alanine, riboflavin, pyridoxamine, guanylic acid, adenylic acid, yeast ribonucleic acid, choline, a flavin component from liver, ribose, inositol, *p*-aminophenyl alanine, pantothentic acid, *p*-aminobenzoic acid, and a strepogenin concentrate could not replace broth or yeast extract in causing an increase of phage in the synthetic medium. This solution did not inhibit phage production when added to the normal broth media.
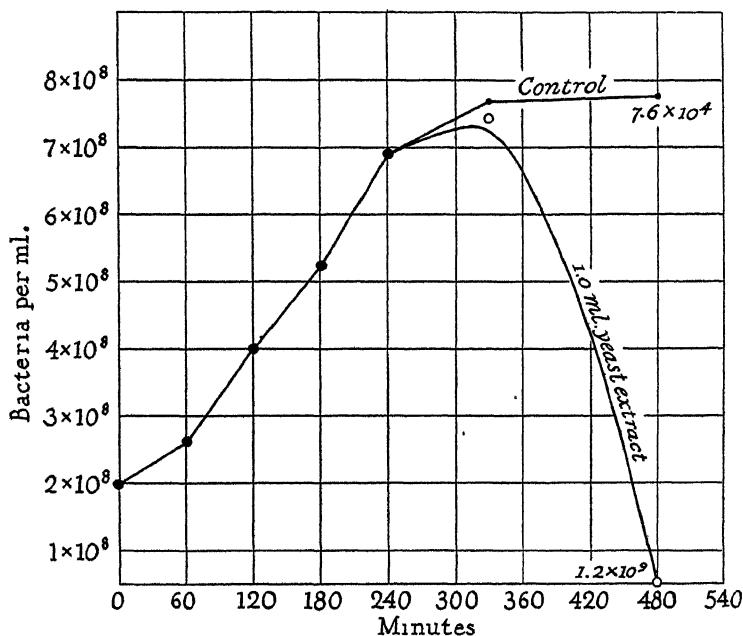
FIG. 2. Effect of yeast extract on phage formation in the synthetic medium. Each tube contained 8.15 ml. of the synthetic medium, 0.15 ml. 0.01 M $CaCl_2$, and 0.9 ml. of phage solution diluted in the synthetic medium. Bacteria were centrifuged, washed once with saline, and suspended in the 8.15 ml. of the synthetic medium. Initial phage plaque counts per ml. were 5.5×10^5. Figures at 480 minutes represent final phage plaque counts per ml.

Work is in progress on the identification of the substance or substances in yeast extract necessary for phage formation.

DISCUSSION

Bacterial concentrations of 2.5 to 3.5 $\times$ 10^8 cells per ml. in broth diluted 1/17 in Locke's solution form phage in the presence of penicillin but form very little phage in the absence of penicillin. The addition of niacin to the above

bacterial concentrations permitted great increases of phage in the absence of penicillin. This result can be interpreted by assuming that penicillin prevents the utilization of niacin by bacteria thus preventing their growth and allowing the phage to utilize niacin. This accounts also for the action of penicillin on bacteria. However, the non-utilization of niacin could also be accounted for by assuming that penicillin prevents some other reaction (3, 4) which inhibited the growth of the organism, and that non-multiplying bacteria did not use niacin. Further work is necessary to decide between these two hypotheses.

Bacteria will grow in the synthetic medium of Fildes but will not form phage unless yeast extract or broth is added. This observation may be extremely important if it could be shown to apply to other viruses.

The results in this paper indicate that there is a continual competition between the bacteria and the phage for essential building elements. Anything which upsets the equilibrium will of course greatly increase the formation of one of the components at the expense of the other. By limiting the amount of a compound essential for virus multiplication, but not necessary for the host, the growth of viruses could be controlled. Since it is well known that the nutritional state of the animal greatly influences the resistance of the host to infection (5–8) further work along the lines outlined above may prove helpful in the control of infectious disease.

By the use of the penicillin system described in this paper, it should be possible to gain a further insight into phage formation by the use of inhibitory metabolic analogues. Such a study will be reported at a later date.

SUMMARY

1. The addition of penicillin greatly increases the production of phage in bacterial suspensions containing 2.5 to 3.5 $\times$ 10^8 cells in 0.4 ml. broth plus 6.6 ml. Locke's solution.

2. Addition of niacin also greatly increases the formation of phage in the above system without the addition of penicillin.

3. The results indicate that niacin is necessary for phage production and that bacteria cannot utilize niacin in the presence of penicillin.

4. *Staphylococcus muscae* will grow in the synthetic medium of Fildes but do not form phage unless broth or yeast extract is added.

5. Phage formation requires the presence of one or more factors, besides niacin, present in broth and yeast extract which are not essential for bacterial growth. Penicillin does not prevent the utilization of the unknown substance or substances by the bacteria.

6. A solution containing biotin, guanine, adenine, β-alanine, riboflavin, uracil, pyridoxamine, guanylic acid, adenylic acid, yeast nucleic acid, choline, *p*-aminobenzoic acid, a flavin component from liver, ribose, thymine, xanthine, folic acid, inositol, *p*-aminophenyl alanine, pantothentic acid and a strepogenin

concentrate cannot replace broth or yeast extraction in increasing phage formation in the synthetic medium of Fildes.

7. The results indicate there is a continual competition between the bacteria and phage for certain essential building elements.

8. The results are discussed in relation to possible methods of control of virus diseases.

Experimental Methods

All methods for measuring and preparing the bacteria and phage are described in the preceding paper (1).

The yeast extract used in this paper was prepared according to Northrop (9).

Addendum.—Experiments concluded after this paper was sent in for publication showed that neither niacin nor the unknown factor is needed for the adsorption of the phage to the bacteria, but that both substances are necessary for the actual multiplication of the phage. A detailed analysis of the effect of the two compounds on phage formation is in progress and will be presented in a later paper of this series.

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BACTERIOPHAGE FORMATION WITHOUT BACTERIAL GROWTH

III. The Effect of Iodoacetate, Fluoride, Gramicidin, and Azide on the Formation of Bacteriophage

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In recent years it has become increasingly apparent that adenosinetriphosphate is the energy source for many types of endergonic reactions. Proof of this statement is found in the need of energy-rich phosphate for such diverse reactions as carbohydrate synthesis (1), uptake of CO_2 by autotrophic bacteria (2), fat metabolism (3), methylation (4), acetylation (5), muscle contraction (6), and perhaps peptide synthesis (4, 7). Consequently the relationship of adenosinetriphosphate (ATP) to phage formation was investigated with the aid of iodoacetate, fluoride, and azide (NaN_3). These substances share the common property of blocking ATP formation by inhibiting certain reactions in the Embden-Meyerhof carbohydrate cycle.

Iodoacetate prevents reaction 1, (8).

(1) Phosphoglyceraldehyde + coenzyme 1 + inorganic phosphate + adenosinediphosphate
$\updownarrow$ triosephosphate dehydrogenase
phosphoglyceric acid + reduced coenzyme + ATP

Fluoride inhibits reaction 2, (9).

(2) 2-phosphoglyceric acid
$\updownarrow$ enolase
phosphopyruvate

The exact mechanism by which azide inhibits ATP synthesis is not known (10).

There is good evidence that staphylococcus forms ATP according to the Embden-Meyerhof cycle and that the usual phosphorylated intermediates are concerned in the metabolism of the organism (11–14).

The system used to study the effect of these inhibitors was the penicillin-treated bacteria described in the previous paper (15). All the inhibitors used prevented multiplication of the bacteria. In the penicillin bacterial system, however, phage formation does not depend on multiplying bacteria; therefore, the inhibitors do not act indirectly on phage formation by inhibiting the multiplication of the bacteria.

RESULTS

The Effect of Iodoacetate, Azide, and Fluoride on Phage Production.—Table I
illustrates that iodoacetate, sodium fluoride, and azide completely inhibit the
formation of phage and also prevent the synthesis of ATP. This effect is
reversible. When the inhibitor was diluted out there was no inhibition of
phage production. That ATP was the phosphorous compound actually being

TABLE I

Effect of Iodoacetate, Fluoride, and Azide on Phage Production and ATP Formation

All tubes contained 5.6 cc. Locke's solution + 0.4 cc. nutrient broth + 0.2 cc. M/2 glucose
+ 1.0 cc. broth containing 160 γ of penicillin and 0.8 cc. phage solution. No phage in sample
5. Inhibitors and penicillin added to bacterial suspension 2 hours before phage. Lysis of
control tube took place at 6 hours. No change in turbidity of samples containing inhibitors.
ATP analysis based on 18.1 mg. of bacterial protein which was determined by micro-Kjeldahl.

Sample	Addition	ATP	Initial phage units	Final phage units
		γ		
1	0.001 M iodoacetate	2	10^6	10^5
2	0.06 M NaF	4	10^9	10^4
3	0.005 M NaN$_3$	3	10^6	10^6
4	None		10^6	10^9
5	None	25		

TABLE II

Comparison of Inorganic Phosphate, Adenosinetriphosphate, Ribonucleic Acid, and Desoxy-
ribonucleic Acid of Normal and Infected Cells

All tubes contained 5.0 cc. broth containing 1 per cent glucose and 1.0 ml. of broth con-
taining 180 γ of penicillin. Infected sample received 1 cc. of phage solution having a titer
of 10^7 phage units per cc. Samples taken for analysis at 3 hours. This was 15 to 30 minutes
before lysis. Ribonucleic acid expressed as γ ribose for total bacterial protein. Colorimeter
readings used for desoxyribonucleic acid values. Analyses based on 11.1 mg. bacterial protein
which was determined by micro-Kjeldahl.

Sample	Inorganic phosphate	ATP	Ribonucleic	Desoxyribonucleic
	γ	γ	γ	
Infected	21	14	360	30
Control	19	13	355	30

measured was indicated not only by the fact that it had phosphate groups
hydrolyzable in N HCl at 100° in 7 minutes, but also that the compound was
precipitated by barium at pH 8.2 in 10 per cent alcohol and would transfer one
of its acid-labile phosphate groups to glucose to form glucose-6-phosphate in the
presence of crystalline yeast hexokinase and magnesium. ATP is the only
known substance that can phosphorylate glucose in the above manner (16).

The Effect of Gramicidin on Phage Formation.—Gramicidin was found to prevent the multiplication of phage. It has been found by Hotchkiss (17) and confirmed in the present work that gramicidin completely prevents the uptake of inorganic phosphate from the medium. The significance of this observation will be discussed later in the paper.

Chemical Analysis of Infected Cell.—Inorganic phosphate, ATP, ribonucleic acid, and desoxyribonucleic acid were determined in normal cells and infected cells (Table II). No differences were detected in any of the compounds between the two types of cells under the experimental conditions. It is very possible that conditions may be found where differences could be shown. Cohen (18) has reported an increased synthesis of desoxyribonucleic acid in *E. coli* cells infected with T_2 phage.

DISCUSSION

Iodoacetate, fluoride, and azide prevent the formation of bacteriophage. Since these substances all inhibit the formation of adenosinetriphosphate, it appears that energy-rich phosphate is needed in the formation of bacteriophage. It has been reported that azide prevents that formation of adaptive enzymes (19). Iodoacetate and azide have also been found to prevent the multiplication of vaccinia virus in tissue culture (20).

Gramicidin prevented the production of bacteriophage. This result is of interest in view of Cohen's (18) report that only the phosphate added to the medium is found in the bacteriophage. As gramicidin completely prevents the uptake of phosphate from the medium it would, according to Cohen's results, prevent the formation of phage. If Cohen is correct, it makes the precursor theory of phage formation less likely, for in this case, one would expect to find some bacterial phosphate in the bacteriophage.

Cohen (18) has shown that only extracellular nitrogen is found in the T_2 phage. In the preceding paper (21) it was reported that multiplying bacteria do not form phage unless accessory substances are added. If the precursor is a normal constituent of cells, one would expect that growing bacteria would form phage without such substances being added. It appears that the phage is directly formed from substances in the media.

Methods

Assay of bacterial suspensions and phage was carried out as described previously (15). Bacterial suspensions were prepared in the same manner as described previously (15).

Chemical Determinations.—The bacterial suspension was centrifuged and washed two times with saline at 0°. The cells were then ground and extracted in a mortar with 5 cc. of 10 per cent trichloroacetic acid plus a little alundum. The residue was reextracted with 5 cc. of 5 per cent trichloroacetic acid. The filtrates were combined and analyzed for inorganic phosphate and ATP. Inorganic phosphate was deter-

mined by the method of Fiske and SubbaRow (22). ATP was determined according to Lohmann and Oppenheimer (23).

The trichloroacetic acid–insoluble precipitate was extracted for nucleic acid by the modification of Schneider's method according to Krampitz and Werkman (24). Ribonuclic acid was determined by the method of McRary and Slattery (25). Desoxyribonucleic acid was determined according to Hoagland *et. al.* (26).

SUMMARY

1. Iodoacetate, fluoride, and azide have been found to prevent the formation of phage and to inhibit the synthesis of ATP by *Staphylococcus muscae*. It is suggested that energy-rich phosphate is needed for the synthesis of phage.

2. Gramicidin prevented the formation of phage.

3. No differences were found between normal bacteria and phage-infected bacteria in the inorganic phosphate, adenosinetriphosphate, ribonucleic acid, and desoxyribonucleic acid content of the cells.

4. The mechanism of phage formation is discussed.

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COLORBLIND VISION

I. Luminosity Losses in the Spectrum for Dichromats*

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I

Nature of Work

In recent years the shape and location of the spectrum luminosity curves of colorblind individuals have been determined with good precision (Pitt, 1935; Hecht and Shlaer, 1936). Fig. 1 presents the average measurements of six deuteranopes and six protanopes, as found by Pitt. The measurements by Hecht and Shlaer on three subjects are in agreement with Pitt's data. Fig. 1 shows that compared to the normal luminosity, the protanope curve is displaced considerably toward the blue, whereas the deuteranope curve is only slightly displaced toward the red.

The measurements in Fig. 1 are drawn in the traditional way of considering each luminosity curve by itself and placing the point of maximum brightness for each at 100 per cent. The question arises as to whether the three curves really have the same height, and if not, what their relative heights are. This is particularly relevant to the problem of what colorblindness is and what relation it has to normal color vision.

Normal color vision is best described in terms of a three receptor system; and colorblindness is often assumed to represent the loss or inactivation of one of these receptor systems. If this is true, then certainly the loss of one of the three receptor systems should show itself in some loss of brightness in the spectrum as experienced by the colorblind. One might expect the colorblind curves in Fig. 1 to have lower maxima than the normal. Abney (1913) drew such lowered luminosity curves for colorblinds. However, these reduced curves were not found from measurements, but were invented in terms of theory (Abney, 1913, page 281).

Because of this total lack of knowledge, we decided to find the relative heights of the three curves in Fig. 1 by measuring the actual visual thresholds of color-

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* A report on these findings was given to the Optical Society of America at its meeting on October 3 to 5, 1946 in New York and to the International Conference on Colour Vision in Cambridge, England, July 28 to 29, 1947.

† Died, September 18, 1947.

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blinds and normals in different parts of the spectrum. The idea is to determine for a given portion of the spectrum the amount of light required at the foveal threshold of normal persons and of colorblind persons, and to do this for different parts of the spectrum so chosen as to cover the spectral range.
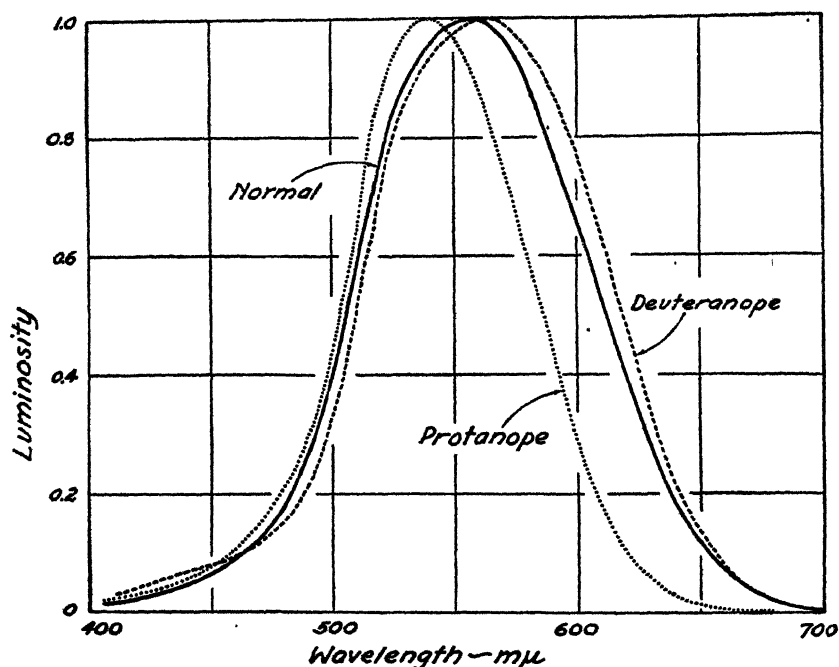
FIG. 1. Spectrum luminosity distributions for normals and colorblinds. The maximum for all curves is arbitrarily put at 100. Data from Pitt (1935).

II

Selection of Subjects

For our measurements we used nine color-normals, six protanopes, and seven deuteranopes, all men between 20 and 35 years of age.

All subjects were tested for their color vision in several ways. The first test consisted in reading the Ishihara pseudoisochromatic plates, "Tests for Colorblindness," 5th edition. Following this, the subject read Stilling's "Pseudo Iso-Chromatische Tafeln," 19th edition. Anyone who read correctly all the plates in these two tests without hesitation was tentatively considered normal. He was then further tested for the Rayleigh equation with an anomaloscope made in our own Laboratory. The equation requires the subject to match a yellow of 575 mμ with a mixture of 555 mμ green and 635 mμ red. A subject was considered normal only when his match was sharp and fell in the narrow range found by us in unpublished studies with many normal persons.

The colorblind subjects were all complete dichromats, not anomalous trichromats. We first chose those persons who made extensive errors in the Ishihara and Stilling tests. Of these we selected only those who in the anomaloscope test were able to match perfectly the yellow of 575 mμ with the green of 555 mμ by itself, and also with the red of 635 mμ by itself. By comparing the relative brightness at match of the yellow and red we classified the subject as protanope or deuteranope; if the red was much brighter (to us) than the yellow he was called protanope (or loosely, red-blind), whereas if at match the red and yellow were equally bright, he was called a deuteranope (or green-blind). The final critical test was always to determine the presence of a neutral point, or white spot, in the spectrum. Only those subjects who matched a sharply located neutral point in the spectrum with whole white light of 5000°K as a standard were considered as complete dichromats, and were used as subjects for the measurements. The neutral point determinations were made with a modified Helmholtz color mixer (Hecht and Shlaer, 1936).

TABLE I

Filters for Isolating Spectrum Regions

Spectral region	Filters	Central λ
		mμ
Blue	C 511 + C 368	469
Blue-green	W 75 + C 978	500
Green	W 74	535
Yellow	W 73 + C 978	579
Red	C 241 + C 978	651

C means Corning glass filter; W means Wratten gelatin-in-glass filter.

III

Apparatus and Procedure

The measurements of the foveal thresholds were made with the original adaptometer designed for just such purposes (Hecht and Shlaer, 1938). In this instrument the intensity of the test light is varied with neutral filters and a neutral wedge, while the color of the light is controlled by appropriate color filters.

Five regions of the spectrum were isolated by means of the filter combinations given in Table I. The transmissions of the individual filters were measured with a photoelectric spectrophotometer (Shlaer, 1938). The transmissions of the filter combinations are shown in Fig. 2. For each filter combination the transmissions of the neutral filters and of the wedge were measured with a Martens polarization photometer. The color temperature of the lamp of the adaptometer was measured with an Eastman color temperature meter and found to be 2700°K.

The absolute brightness of the test field in white light was measured directly at a chosen wedge setting with a Macbeth illuminometer placed at the exit pupil of the adaptometer. Knowing this brightness, and knowing the transmissions of the neutral and color filters, the slope of the wedge, and the color temperature of the lamp, one can

compute precisely the spectral composition and the relative energy content of the test fields used in the measurements.   Since these calibrations are merely detailed steps in the computation of the final results, they need not be given here.

Observations were monocular, and the subject fixated a tiny red point reduced to the lowest possible brightness by the subject himself.   The circular test field was central, and was 1° in diameter.   Exposures were in flashes of ⅛ of a second, and were controlled by the subject.   The experimenter arranged the wedge and the filters, and the subject manipulated the shutter when he was ready to observe the flash.   He merely reported the presence or absence of light.
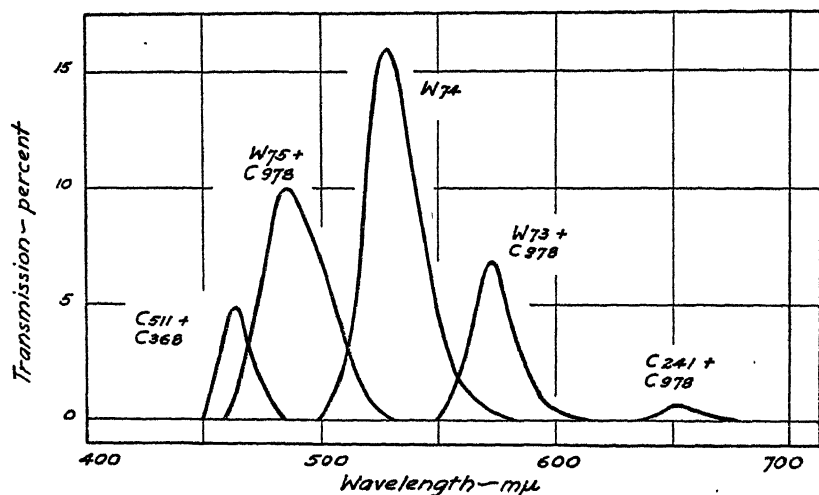
FIG. 2 Transmission of filter combinations for isolating parts of the spectrum.   C is Corning and W is Wratten.

The natural pupil was used.   Because the subjects were all dark-adapted, the pupil was maximal.   Variations in the pupil size at maximal pupil opening are of only trifling influence on cone vision.   This is due to the Stiles-Crawford phenomenon (Stiles and Crawford, 1933) which renders the light passed by the peripheral portion of the expanded pupil extremely inefficient in stimulating the cones.

Cone adaptation is usually over in 10 minutes of darkness, even after exposure to very bright light.   We allowed our subjects at least 15 minutes in the dark after a preliminary stay in the laboratory before beginning observations so that cone dark adaptation was complete.

An experimental session consisted of determining three times in succession the final threshold for seeing the central 1° field at the five selected positions of the spectrum. We always started with the blue end of the spectrum and worked toward the red. Most of our subjects came for four such sessions.   During the first session the measurements were recorded but not counted because we considered the period as one of instruction and practice.   The remaining three separate sessions constituted the final measurements.

IV

Relative Luminosities

Our interest in the present study is to determine the differences in foveal threshold between colorblinds and normals. To establish the normal base-line, we have averaged the measurements for the nine color-normals at each spectrum locus, and have put the average threshold value equal to unity.[1] Table II then shows the logarithmic deviations of the nine observers from the average values and gives some idea of the individual variations encountered. .

In the same Table II the thresholds of the protanopes and of the deuteranopes are also shown as deviations from the average color-normal. From these deviations one can assess the magnitude and the validity of the average differences between normal and colorblind persons.

It is apparent from Table II that beginning with the blue and going toward the red, the threshold of the protanope, compared to the normal, steadily rises in the spectrum. The two thresholds are practically identical in the blue, but the protanope threshold is well over 1 log unit greater than the normal in the red. The deuteranope threshold is also very nearly normal in the blue; but it definitely rises in the green and remains at about that level through the rest of the spectrum. The average measurements are shown in Fig. 3 where the normal is represented by a horizontal line and the thresholds of the two kinds of colorblinds are shown displaced above the normal the appropriate logarithmic distance.

In order to see what these measurements actually do to the shapes and positions of the colorblind luminosity curves, we need to place them in relation to the normal luminosity distribution in the spectrum. This is done in Fig. 4 with relation to the color-normal curve taken from Wald's recent study (Wald 1945). The reason for choosing Wald's data is that they were secured in essentially the same way as ours, that is, by measurements of the energy threshold of a 1° field in the central fovea after complete dark adaptation. However, with only minor differences Wald's normal curve is practically the same as the standard luminosity curve for the normal eye which has been used for years (Gibson and Tyndall, 1923).

In Fig. 4 at the selected five points on the spectrum, the average logarithmic

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[1] The actual averages of the brightness thresholds for the nine normal subjects for the spectrum loci 469, 535, 579, and 651 mμ are respectively 6.13, 6.15, 6.13, and 6.17 in log micromicrolamberts. These threshold values are essentially identical, as they should be. However, the average threshold for 500 mμ for the same subjects is 6.32 in log micromicrolamberts, that is, about 0.15 log unit higher than it should be. Recalibration of all the filters and the wedge, and reworking of all the computations yielded no change in these values. Since we are interested only in differences between normal and colorblind observers this curious deviation has no relevance. ·

differences found in Table II are drawn for the protanope and deuteranope as displacements downwards from the normal curve of Wald. Through these

TABLE II

Foveal Thresholds of Colorblinds Compared to Normal for 1° Field

The average normal threshold is put at 1. All values are given as logarithmic deviations from the average normal (log 1 = 0).

Color vision	Subject	Deviation from normal mean in log units at				
		469 mμ	500 mμ	535 mμ	579 mμ	651 mμ
Normal	1	−0.04	−0.01	+0.06	+0.06	−0.03
	2	+0.06	+0.05	+0.02	+0.02	+0.15
	3	−0.04	−0.07	+0.01	+0.08	+0.07
	4	+0.10	+0.01	−0.09	−0.12	−0.09
	5	+0.05	+0.11	+0.08	+0.06	−0.03
	6	−0.03	−0.05	−0.14	−0.05	−0.01
	7	−0.12	−0.15	−0.11	−0.13	−0.10
	8	+0.01	+0.07	+0.07	+0.02	+0.04
	9	−0.01	+0.01	+0.08	+0.09	0.00
	Average.......	0	0	0	0	0
Protanope	1	+0.03	+0.10	+0.29	+0.63	+0.87
	2	−0.03	+0.01	+0.21	+0.49	+1.37
	3	+0.11	+0.11	+0.15	+0.29	+1.06
	4	+0.08	+0.14	+0.19	+0.23	+1.21
	5	+0.11	+0.14	+0.14	+0.20	+1.05
	6	+0.06	−0.05	+0.14	+0.15	+1.11
	Average.......	+0.06	+0.08	+0.19	+0.33	+1.11
Deuteranope	1	−0.12	−0.02	+0.09	+0.07	+0.04
	2	+0.19	+0.30	+0.30	+0.30	+0.34
	3	+0.13	+0.11	+0.33	+0.26	+0.21
	4	+0.16	+0.11	+0.19	+0.20	+0.23
	5	+0.08	+0.11	+0.29	+0.20	+0.03
	6	+0.01	+0.10	+0.21	+0.23	+0.21
	7	+0.01	+0.08	+0.22	+0.28	+0.26
	Average.......	+0.07	+0.11	+0.23	+0.22	+0.20

displaced points we have drawn smooth curves. Fig. 4 is therefore the same as Fig. 3 except that instead of the normal curve being rectified, it is now presented in its real spectral form. Fig. 4 shows unequivocally that for both types of colorblindness there occurs not merely a shift of maximum or a change in shape of the curve, but a real loss of luminosity in the spectrum.
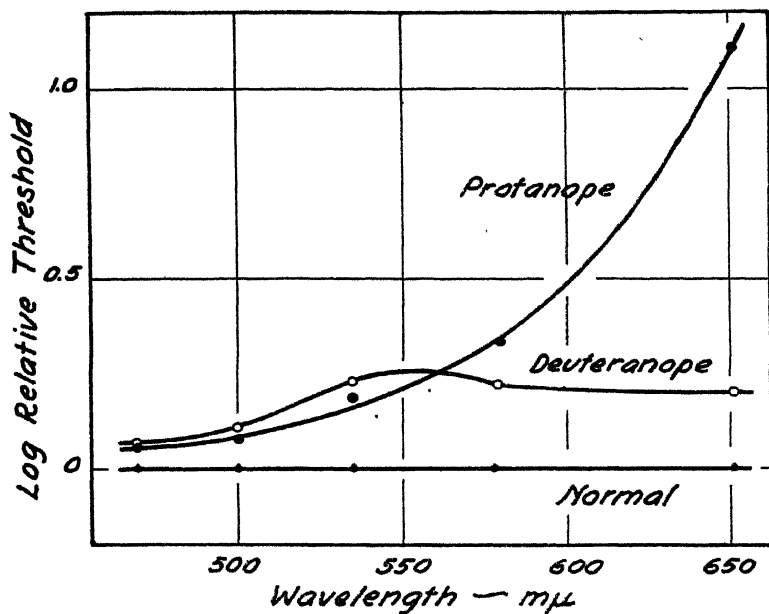
FIG. 3. Average foveal thresholds of six protanopes and seven deuteranopes compared to nine normals. Test field is circular and 1° in diameter.
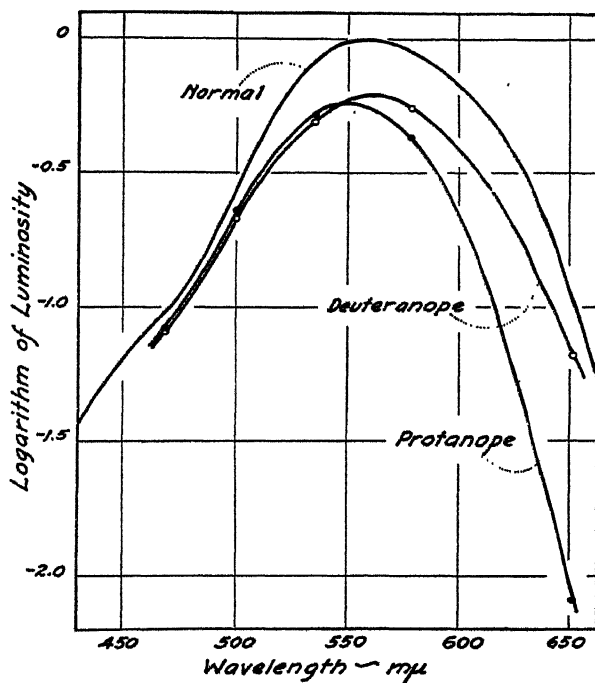
FIG. 4. Real luminosity distributions in the spectrum of normals and dichromats.

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To determine the precise amount of this loss, we have plotted the data in arithmetical form in Fig. 5. The area under such an arithmetical luminosity curve represents the total brightness of an equal energy spectrum. We have measured with a planimeter the relative areas under the three curves. If the area under the normal curve is put equal to 100, the area under the protanope curve turns out to be 51.0, whereas the area under the deuteranope curve is 61.2.
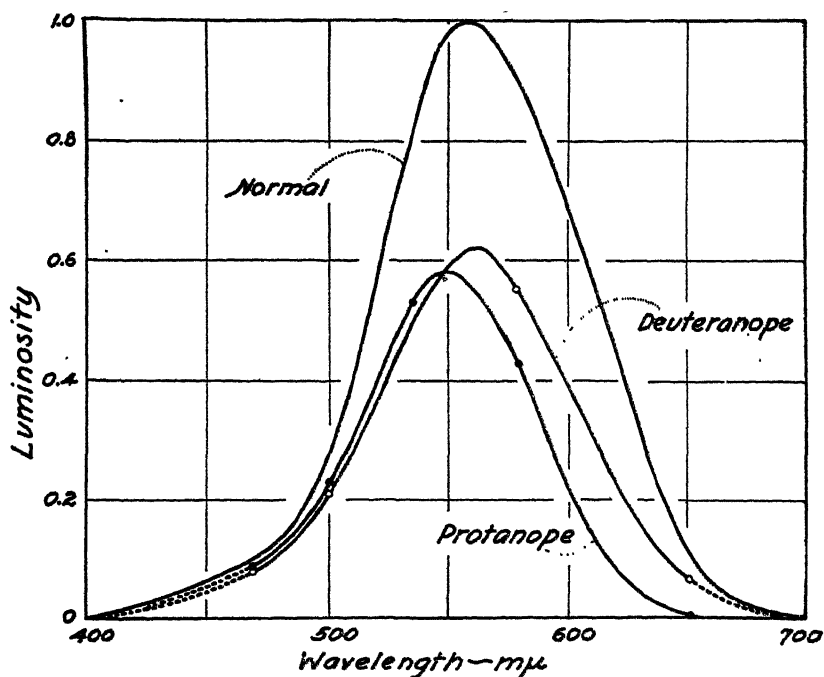
FIG. 5. Arithmetical luminosity distributions in the spectrum to show the real heights of the colorblind curves compared to the normal. The areas under the curves indicate the total brightness of an equal energy spectrum. When the normal area is 100, the protanope area becomes 51.0 and the deuteranope area becomes 61.2.

Evidently, compared to the normal, the protanope loses almost one-half the luminosity of the spectrum while the deuteranope loses almost two-fifths the luminosity of the spectrum. These losses are large, and must have meaning for color vision theory.

V

Color Vision and Colorblindness

There is no completely adequate theory of color vision. However, the one reasonable basis for such a theory has consistently been Young's notion that

there are three receptor systems in the retina (Young, 1807) which may be designated as B, G, and R to indicate their essentially qualitative uniqueness in yielding respectively blue, green, and red sensations when brought into action by light. Each receptor system produces only the sensation unique for it, regardless of the part of the spectrum which sets it into action, and the sensations produced by various parts of the spectrum result from the combined action of these three systems in different degrees. Certain combinations produce specific effects. Thus the combined actions of the G and R systems result in the unique sensation of yellow, while the combined actions of B, G, and R result in the unique sensation of white (cf. Hecht, 1928). Moreover, the action of the receptors contributes brightness as well as color, and the brightness contributions of the three systems are strictly additive.

Young supposed that colorblindness is due to the loss of one of these three receptor systems. This supposition is still held in one form or another (Pitt, 1944).

Our present measurements support this simple and direct formulation for colorblindness. If a protanope has lost the R receptor system, he should lack not only its color effects but also its brightness contribution, and the loss should correspond to the contribution which the R receptor system makes to the normal luminosity of the spectrum. Since our data show that the protanope suffers a loss of 49 per cent in spectrum luminosity, it would seem that the R system normally contributes 49 per cent of the brightness of an equal energy spectrum. In the same terms, the loss of 39 per cent of spectrum luminosity by the deuteranope corresponds to his loss of the G receptor system, and would indicate that the G system contributes 39 per cent of the normal spectrum brightness.

Since the total brightness is supplied by $B + G + R$, and since $G + R$ together contribute 88 per cent, the remaining 12 per cent must represent the contribution of the B receptor system alone. It follows from this reasoning that if tritanopia—the third form of colorblindness—results from the loss of the B receptor system, it should involve the loss of only 12 per cent of the brightness of an equal energy spectrum. No measurements now exist to judge of this, and it therefore represents a specific prediction in terms of Young's idea of colorblindness.

The relative brightness contributions of the B, G, and R systems as 12, 39, and 49 per cent respectively are roughly the same as many previous estimates of the contributions of these three systems (Ives, 1923; Wright, 1929–30). In general the G and R systems have been given approximately equal weight in normal luminosity, whereas the B system has most often been evaluated as even less than 10 per cent. The present measurements do not support the notion (Hecht, 1931) that the three receptor systems contribute equally to the brightness of the spectrum.

Even though the simple idea that colorblindness is due to a loss of one receptor system accounts for our luminosity measurements, it does not account for other equally important aspects of colorblindness, particularly the color sensations.   If in normal vision the varying degrees of activity of all three systems, B, G, and R, produce all the varied color sensations, then the loss of one system should reduce the number and alter the quality of these sensations. For example, if the R system is lost, no red sensations should be possible.   Loss of the R system should leave the spectral gamut as made up only of blue and green sensations.   Moreover, since yellow occurs from the combined activities of the G and R systems, the protanope should have no yellow sensation, and indeed no white sensation either because the action of all three systems is required for this effect.

A similar situation must obtain for the deuteranope also.   Having lost the G system, he should see neither yellow nor white as we do.   Moreover, the tritanope, because of his lost B system, should also be unable to see white as do normals; to him it should appear yellow because of the action of the G and R systems alone.

None of these consequences of the loss of a receptor system is true.   Color-blind persons, beginning with Dalton (1798) who described his sensations with great clarity, insist that they see white as uniquely colorless.   Their insistence is confirmed by those occasional individuals who are normal in one eye and colorblind in the other.   The best described case is that of a tritanope (Dieter, 1927) who on comparing the spectrum and other colors with his two eyes described white as the same with both eyes.   In particular, the neutral points at 575 mμ and 415 mμ were unequivocally described as white.   Other instances of monocular colorblindness (von Kries, 1919) bear out these facts about white.

Moreover, in terms of the loss of a receptor system, neither the deuteranope nor the protanope can have the sensation of yellow.   Yet these dichromats consistently describe the spectrum as made up of two hues: blue on the short-wave side of the white neutral point, and yellow on the long-wave side of the neutral point.   In addition they record that these two hues are most saturated at the extremes of the spectrum, and gradually become unsaturated toward the neutral point which is completely unsaturated as white.   In fact, colorblinds make wavelength discriminations in the spectrum on either side of the neutral point not in terms of hue but in terms of saturation (Hecht and Shlaer, 1936).   These descriptions are corroborated by von Kries' monocular colorblind.   In short the sensory reports given by dichromats cannot be accounted for by Young's idea of the simple loss of one receptor system.

The sensations reported by colorblinds may be accounted for by an alternate proposal first suggested by Fick (1879).   According to this proposal, colorblindness involves the transformation of the spectral sensitivity of one receptor system into that of one of the other receptor systems.   For example, suppose that the R system is changed so that its sensitivity in the spectrum becomes

identical with the G system. The altered R receptor system and the normal G system will now be equally stimulated by light of any part of the spectrum. This applies only to the outermost light-receiving end of the receptor elements. The rest of the altered R system beyond the receptor elements will remain as before. Thus regardless of the light which it receives, it will still transmit impulses which will result in the production of the unique red sensation. Such an eye will not be able to discriminate hues in the spectrum on the long-wave side of the neutral point because this whole region will be yellow of different degrees of saturation.

This formulation of colorblindness accounts for the sensations which color-blinds have, and for the errors and confusions which they make. Unfortunately it is not supported by our present measurements. For instance, if the R system is not lost, but its sensibility distribution in the spectrum is merely altered, then there should be no loss in the total spectrum luminosity of protanopes. Yet our measurements show a 50 per cent loss. And a similar situation obtains for a deuteranope, whose loss is almost 40 per cent. Thus the idea of a simple loss of one receptor system accounts for the luminosity measurements but completely fails to account for the sensations; whereas the idea of a transformation of one receptor system into another accounts for the sensations, but cannot encompass the luminosity loss data.

Pitt's recent (1944) proposal that protanopia represents a simple loss of R whereas deuteranopia represents a transformation of G into R does not resolve this dilemma. Both types of colorblind show a loss of luminosity, and the sensations of the protanope still remain unaccounted for by a mere loss of receptor system.

We thus have two mutually exclusive suggestions for the basis of colorblindness each of which describes only one aspect of the phenomenon. Clearly, a new formulation for colorblindness is required which will combine the virtues of both ideas and eliminate their contradictions.

<div align="center">SUMMARY</div>

1. Measurements have been made of the dark-adapted foveal threshold of normal and colorblind persons in five parts of the spectrum using a $1°$ circular test field.

2. Compared to normals, protanopes (red-blinds) show an elevation of the threshold which increases slowly from blue to yellow and rises rapidly thereafter until in the red the threshold is more than ten times as high as normal. Deuteranopes (green-blinds) do not show so high an elevation, their maximum in the green being only about 70 per cent above normal.

3. These threshold elevations correspond to luminosity losses in the spectrum. For the protanope the total loss in the spectrum is nearly one-half of the normal luminosity; for the deuteranope it is nearly two-fifths of normal.

4. Such losses support the idea that colorblindness corresponds to the loss of

one of the three receptor systems usually postulated to account for normal color vision. However, the color sensations reported by colorblind persons, especially monocular colorblinds, do not support the idea of a lost or inactivated receptor system. A fresh explanation for colorblindness is called for to reconcile these conflicting kinds of evidence.

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INTERACTIONS BETWEEN ENZYME-FORMING SYSTEMS DURING ADAPTATION*

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I

INTRODUCTION

Resting suspensions of viable yeast cells can acquire, on incubation with the proper substrates, enzymatic activities not possessed previous to the incubation. This phenomenon provides an almost unique opportunity for the experimental control of cellular enzymatic constitution. An analysis of the factors involved in determining the appearance and disappearance of enzymatic activities thus becomes feasible and pertinent.

Previous publications (1–3) have discussed the significance of the available data on enzymatic adaptation for the problem of genic control over enzyme formation. It is evident, however, that before much progress can be made in elucidating the genetic mechanism, more information must be obtained on the biochemical and physiological details of how cells form, maintain, and destroy the enzymes found in the cytoplasm. With such information available one might reasonably hope to arrive at a rational prediction of where in the chain of reactions leading to the formation of an active enzyme system, the gene can exert its influence.

An adequate solution to the problem of active enzyme formation must ultimately answer questions concerning the origin of the protein which constitutes the enzyme and the nature of the energy required for the transformation of inactive protein into a type which possesses the requisite catalytic activity.

Both aerobic and anaerobic metabolism can supply (4, 5) the energy necessary for the emergence of the enzyme systems involved in the adaptive fermentation of galactose and maltose by yeast suspensions. The present paper concerns itself primarily with investigations into the relationship between enzymatic adaptation and the nitrogen metabolism of the yeast cell.

Ever since the classic investigations of Dienert (6) on galactose adaptation in yeast it has been known that it is possible for washed yeast suspended in phosphate buffer to adapt to the fermentation of galactose. Some authors have denied that this is possible. Thus von Euler and Nilsson (7) claimed

that adaptation to galactose will not occur in the absence of added "Z" factor. A few of the strains examined in this laboratory behave like many bacteria in being unable to adapt unless they are suspended in a complete medium. However, with the vast majority of the strains examined in this laboratory our experience agrees with that of Dienert. Adaptation can proceed without requiring any exogenous supplements other than the adapting substrate.

It is of some interest to note that adaptation of washed suspensions of bacteria has been obtained by Pollock and Knox (8) and more recently by Pollock (9) in the case of nitratase in *Bacterium coli*. It would appear therefore that ability to adapt under these conditions is not a unique property of the yeasts.

This capacity of yeast cells to adapt enzymatically in the absence of exogenous nitrogen raises certain questions concerning the origin of the protein involved in the formation of the new enzyme. Some of the problems posed may be phrased in the following terms: (1) Does a storage of enzymatically indifferent protein exist upon which the cell can draw for the formation of a new enzyme? (2) What happens to existent enzymes when a cell is induced to form a new one? (3) Is the course of the adaptation changed by providing an exogenous source of nitrogen?

An attempt is made in the present paper to answer these and related questions for the cases of galactose and maltose adaptation with yeast.

II

Materials and Methods

(a) *Yeast Strains.*—The yeast strains employed here are the same as those used in previous investigations. The majority of the experiments were done with two diploid strains of *S. cerevisiae*, Al and Kl. Unless otherwise specified 48 hour cultures were used. These stock cultures are grown at 29°C. and are transferred every 48 hours.

(b) *Media.*—The culture media was made by adding the following to 1 liter of water; 2 gm. of autolyzed yeast extract powder, 5 gm. of bacto-peptone, 1 gm. $(NH_4)_2SO_4$, 2 gm. of KH_2PO_4, 0.25 gm. of $MgSO_4$, 0.13 gm. of $CaCl_2$, 7 cc. of 50 per cent Na lactate, and 60 gm. of dextrose. After being brought to a boil, the mixture is cleared by filtration.

(c) *Manometric Measurements.*—All measurements were done at 30.2°C. with standard Warburg apparatus. The anaerobic CO_2 production was determined by replacing the air with nitrogen. Inaccuracies due to retention of CO_2 were reduced by using $M/15$ KH_2PO_4 as the suspending medium. Unless otherwise specified the measurement of fermenting capacity with respect to a given substrate was done with the latter at 4 per cent. Enzyme activity was always measured as $Q_{CO_2}^{N_2}$ (cubic millimeters of CO_2 per hour per milligram, anaerobic) in the presence of corresponding substrates.

(d) *Standard Suspensions.*—Suspensions of the desired density were prepared with the aid of a colorimeter calibrated so that readings could be interpreted in terms of

milligrams of dry weight of yeast per cubic centimeter of suspension. In preparing suspensions for adaptations the yeast cells from a 48 hour culture were centrifuged away from the culture medium in 50 cc. tubes. After allowing the medium to drain out, $M/15$ KH_2PO_4 was carefully poured down the side so as not to disturb the cells packed on the bottom, and was then poured out and allowed to drain. This step serves to wash the medium from the sides. This entire procedure was repeated once more. The cells were then resuspended in $M/15$ phosphate and adjusted to the desired density.

(e) *Adaptations.*—Adaptations, unless otherwise specified, were carried out with suspensions in $M/15$ KH_2PO_4 in the presence of 4 per cent of the adapting substrate. The adapting suspensions were shaken in flasks immersed in a bath held at 30°C. In those cases in which adaptations under anaerobic conditions were desired they were carried out in Warburg flasks in which the air was replaced with nitrogen.

<div align="center">III</div>

<div align="center">EXPERIMENTAL RESULTS</div>

(a) The Effect of Exogenous Nitrogen on Aerobic and Anaerobic Adaptation

Cultures adapted to galactose by growth in its presence in a complete medium invariably attain much higher (*ca.* twofold) levels of enzyme activity than those observed in cultures which are adapted by incubation with substrate in phosphate buffer. Whether the presence of exogenous nitrogen is responsible for the higher activity in complete medium was tested in various ways. Standard 48 hour cultures grown in glucose medium were dissimilated for various periods of time and their ability to adapt aerobically to galactose fermentation with and without exogenous nitrogen examined. Dissimilated cultures were employed in order to compare cultures with varying abilities to adapt (5). In all of these experiments the incubation was carried out in the presence of 4 per cent galactose in shaking flasks at 30.2°C. Samples were removed at intervals and washed with chilled $M/15$ KH_2PO_4. The washed cells were resuspended in their original volume and their ability to ferment galactose anaerobically determined.

In order to examine the adaptation in the presence of nitrogen uncomplicated by extensive cell division the amount of nitrogen (as $(NH_4)_2SO_4$) added corresponded in each case to 50 per cent of the nitrogen content of the yeast suspension. This would permit at the most of an increase in cell number of 50 per cent. Actually, under these conditions, little if any budding is seen and the increase in density of the suspension in the period allowed for the experiment rarely exceeds 5 per cent.

Some typical results obtained with dissimilated cultures of strain K are given in Fig. 1. As is seen from curve (A) this culture was dissimilated long enough so that it showed no evidence of adaptation after more than 7 hours' incubation with galactose. The same suspensions, however, (Fig. 1, curve (B)) exhibited galactozymase activity within 4 hours if incubated with exogenous

nitrogen. Curves (C) and (D) compare the effect of nitrogen on the adaptability of a moderately dissimilated culture. As may be seen from curve (B), the addition of nitrogen greatly accelerates the adaptive process and also permits the attainment of much higher levels of maximal activity.

The question of whether nitrogen has a similar effect on adaptation under anaerobic conditions was also examined. It has been shown previously (4) that enzyme adaptation will occur anaerobically providing the culture is pre-
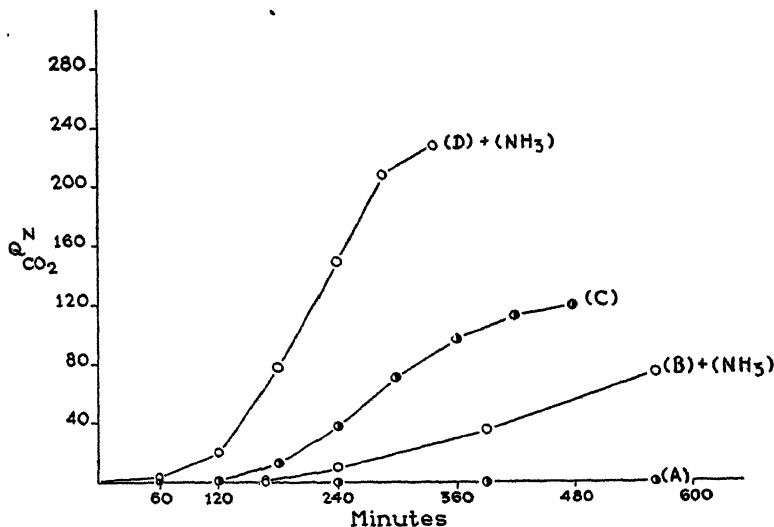
FIG. 1. The effect of added $(NH_4)_2SO_4$ on aerobic adaptation to galactose. (A) Control dissimilated culture; (B) dissimilated culture plus added nitrogen; (C) control partially dissimilated; (D) partially dissimilated plus added nitrogen.

incubated with substrate aerobically until an amount of enzyme is formed which will permit adequate anaerobic utilization of the adapting substrate. This provides the necessary energy for the completion of the adaptive process.

The results of experiments examining the effect of added nitrogen on this type of anaerobic adaptation are given in Fig. 2. The aerobic preincubation period was varied to permit a comparison of the magnitudes of the effects on cultures which were allowed to reach different levels of enzymatic activity. Curves (A) and (B) experienced no previous aerobic incubation. Under such circumstances no anaerobic adaptation is observed in the control (curve (A)) and the presence of exogenous nitrogen has no observable influence. When however the enzymatic activity is built up to 14 by aerobic preincubation subsequent anaerobic contact with substrate leads to further increase of enzyme activity as is seen from curve (C). Furthermore, the effect of added nitrogen begins to make itself apparent by a small but definite increase in rate of formation (curve (D), Fig. 2). The difference between anaerobic adaptation with

and without exogenous nitrogen is greatly magnified if more enzyme activity is induced before instituting anaerobiosis. This is clearly demonstrated by curves (E) and (F) of Fig. 2.

These experiments would seem to indicate quite definitely that the mere presence of the ammonium salt is not *per se* sufficient to accelerate the adaptive process. The concomitant existence of an active metabolism seems to be required for the exogenous nitrogen to exert its influence. Other experiments confirm this finding. Previous experiments (5) have demonstrated that in addition to the procedure described above, another method may be used to
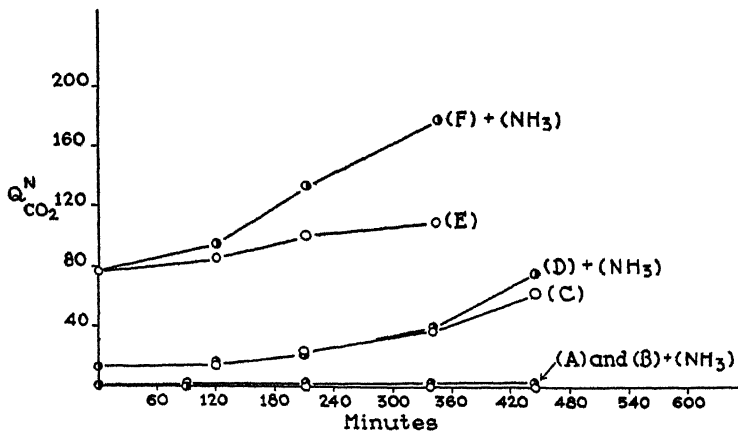
FIG. 2. The effect of added $(NH_4)_2SO_4$ on anaerobic adaptation to galactose. (B), (D), and (F) have nitrogen added; (A), (C), and (E) are the corresponding controls without nitrogen. The initial values of the three groups were attained by previous aerobic incubation before the beginning of the experiment.

obtain adaptation to either galactose or maltose under anaerobic conditions. This latter method employs the relatively simple device of supplying a small amount of some fermentable substrate (*e.g.*, glucose, fructose, or mannose) with the adapting substrate.

The fermentation of the added zymohexose furnishes the requisite energy for the adaptation to proceed anaerobically. The general result obtained is given by curve (B) of Fig. 3. Here 4 mg. of glucose were added simultaneously with the galactose. The vertical line indicates the point at which the amount of CO_2 released is equivalent to the amount of glucose added. Any CO_2 evolved in excess of this must therefore arise from the fermentation of the adaptive substrate, galactose. It is apparent that under the combined influence of the metabolized glucose and the adapting substrate, some adaptive enzyme is formed and subsequently increases on further incubation. Curve (A) records the progress of the same type of experiment except that exogenous nitrogen (50 per cent of cell content) was added simultaneously with the glucose-galac-

tose mixture. It is clear that more enzyme is formed during the early period since (A) does not fall as low as (B) and furthermore, the subsequent appearance of enzyme is more rapid. Curve (C) of Fig. 3 illustrates what occurs when the glucose is added first and the ammonium sulfate and galactose introduced after all CO_2 evolution from the added glucose has ceased. Again it is clear that the exogenous nitrogen must be present during active metabolism in order to produce an effect on the adaptive process. This same experiment further exemplifies the principle previously found (5), that the adapting substrate must
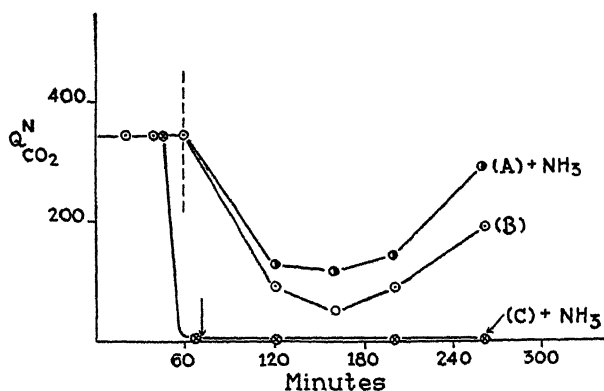
FIG. 3. The effect of exogenous nitrogen on anaerobic adaptation to galactose in the presence of a fermentable substrate. (B) is the nitrogen-free control of (A). (C) is a control with nitrogen in which the adapting substrate, galactose, was added after all the added glucose was fermented. The arrow indicates the point at which the galactose was added.

also be present during the period of active metabolism if the energy generated is to be directed towards adaptation.

The experiments reported here leave little doubt that an exogenous source of nitrogen can stimulate the formation of adaptive enzymes whether the adaptation is occurring aerobically or anaerobically. It is equally evident that such stimulation is found only when the cell is actively metabolizing. It seems likely that this latter fact is connected with the inability of non-metabolizing cells to assimilate nitrogen. Further evidence on this will be given in a subsequent paper which will present data on inhibition of enzyme formation.

The data reported in the present section deal only with adaptation to galactose in a single strain. Similar results have been obtained with other strains and with adaptation to maltose fermentation. While quantitative differences were observed when other strains were used or when other enzyme systems were studied, no fundamental variation from the findings reported above was noted.

(b) *Interaction between Adaptive Enzymes*

In order to obtain some information on the interaction of the cellular enzymes during the adaptive process an examination was made of the influence of one adaptive enzyme on the formation of another. Experiments were performed which compared adaptability to a given substrate subsequent to being adapted to another one. In practice, these experiments were carried out by adapting cells to galactose and then adapting to maltose and *vice versa*. The behavior of

TABLE I

Interaction between Adaptive Enzymes in Phosphate Buffer

The numbers represent $Q_{CO_2}^N$ with substrate corresponding to enzyme being induced attained after 3 hours of incubation.

Strain	Experiment No.	Enzyme being induced	Adaptation with culture adapted to				
			(1) Glucose	(2) Galactose	Per cent of (1)	(3) Maltose	Per cent of (1)
K	1	Galactozymase	40	—	—	38	95
	2	"	42	—	—	41	98
	3	"	39	—	—	39	100
A	1	"	34	—	—	20	59
	2	"	30	—	—	20	67
	3	"	27	—	—	16	59
K	1	Maltozymase	159	61	38	—	—
	2	"	165	53	32	—	—
	3	"	160	58	36	—	—
A	1	"	184	87	47	—	—
	2	"	193	91	47.	—	—
	3	"	186	74	40	—	—

unadapted (glucose-grown) controls was used for comparison. All of these experiments were carried out in $M/15$ KH_2PO_4 in the absence of any exogenous nitrogen. The results of such experiments on two strains are presented in Table I. Column (1) records the enzyme activities attained by suspensions which have not been previously adapted to either maltose or galactose. Comparison of columns (1) and (3) shows that when galactozymase is being induced in strain K it is immaterial whether the culture has been previously adapted to maltose or not; the same galactozymase activities are arrived at in 3 hours in either case. However, with strain A, a suspension which has already formed maltozymase finds it more difficult to adapt to galactose. As may be seen from the last column of Table I, only about 63 per cent of the control galac-

tozymase activity is reached by cells of strain A previously adapted to maltose. In the reverse experiments, galactozymase was induced first and subsequent adaptability of maltose was examined. In these we see that for both strains a drastic decrease in the attained maltozymase activity results from a previous adaptation to galactose.

From these results it is evident that the initial enzymatic constitution can influence the cell's ability to form other enzymes. The interaction is obviously not equivalent in both directions. Galactozymase apparently has a greater influence on maltozymase formation than *vice versa*. It is impossible to say at what level the interaction is occurring. But, that it involves nitrogen metabolism is clear from the experiments recorded in Table II. In these experi-

TABLE II

Interaction between Adaptive Enzymes in the Presence of Exogenous Nitrogen

The numbers represent $Q_{CO_2}^N$ with substrate corresponding to enzyme being induced.

	Strain A				
	Adaptation with culture adapted to				
Enzyme being induced	(1) Glucose	(2) Galactose	Per cent of (1)	(3) Maltose	Per cent of (1)
Galactozymase	82	—	—	81	99
	91	—	—	92	101
Maltozymase	283	244	86	—	—
	294	237	83	—	—

ments exogenous nitrogen was present during the adaptation to the second substrate. The presence of exogenous nitrogen completely abolishes the interaction when galactozymase is forming in the presence of maltozymase. The reverse interaction is also greatly diminished in intensity. With exogenous nitrogen about 85 per cent of control maltozymase activity is reached in a galactose-adapted cell as compared with the 45 per cent observed (Table I) in the absence of the exogenous nitrogen.

The severity of the effect of adaptation to galactose on the maltozymase content of a cell can best be seen by following the maltose-fermenting system during the adaptation to galactose. For this purpose, fully adapted maltose cultures, washed and suspended in phosphate buffer, were incubated aerobically with galactose and samples withdrawn at intervals for galactozymase and maltozymase activity determinations. To determine whether exogenous nitrogen had any effect on the interaction a similar suspension was prepared containing in addition to the galactose a source of nitrogen ($(NH_4)_2SO_4$) equivalent in amount to 50 per cent of nitrogen content of the cells present. Table III records the

results of such experiments. Within 1.5 hours a culture adapting to galactose loses 80 per cent of its maltozymase activity and at the end of 3 hours only 3 per cent of the original activity remains. The presence of exogenous nitrogen affords some protection to the maltose-fermenting enzyme system but does not prevent the drop.

All the experiments thus far described deal with enzymatic interactions when only one of the adapting substrates was present at a time. It was of some interest to examine what happened to the nature of the interaction if instead of performing the adaptations serially, simultaneous adaptations to the two substrates were made.

In these experiments suspensions in $M/15$ KH_2PO_4 were incubated aerobically in a mixture of equimolar (0.17) concentrations of galactose and maltose.

TABLE III

The Effect of Adaptation to Galactose on the Maltozymase Activity of a Maltose-Adapted Culture

The numbers represent $Q_{CO_2}^{N}$ values with substrates corresponding to enzymes being tested.

Time	Without exogenous nitrogen		With exogenous nitrogen	
	Maltozymase	Galactozymase	Maltozymase	Galactozymase
hrs.				
0	350	0	350	0
1.5	69	12	82	21
3.0	12	41	31	79

Samples were removed at intervals and galactozymase and maltozymase determinations made on them. Control adaptations with portions of the same suspension were run in parallel with each substrate separately at the same concentration as obtained in the mixture. Fig. 4 gives the results of such an experiment. Curves (B) and (D) represent the control suspensions in which adaptation was occurring in the presence of only one of the two substrates. Curve (B) is the galactose adaptation and (D) the maltose adaptation. Curve (A) records the increasing galactozymase content of the experimental flask in which the cells were in contact with both substrates. Curve (C) of the same figure gives the results obtained when the maltozymase activity was measured in this same suspension. From a comparison of curves (A) and (B) the presence of maltose has little if any effect on the progress of the adaptation to galactose. However, the presence of galactose has a severely inhibitory effect on the formation of maltozymase as may be seen from a comparison of curves (C) and (D). While some maltozymase can form in the galactose-maltose in the early part of the curve, the level observed is far lower than that attained in the control suspension (D) which is adapting only to maltose. Further, the progressive increase in galactozymase content is paralleled by a sharp drop in the

maltozymase attained before the appearance of very much galactozymase activity.

The ability of maltozymase to appear in the early phases of the simultaneous adaptation is probably connected with the relatively great difference in speed of adaptation with respect to the two substrates. A comparison of curves (B) and (D) shows that maltose adaptation has a much shorter lag period and much higher formation rate than adaptation to galactose. However, once the galac-
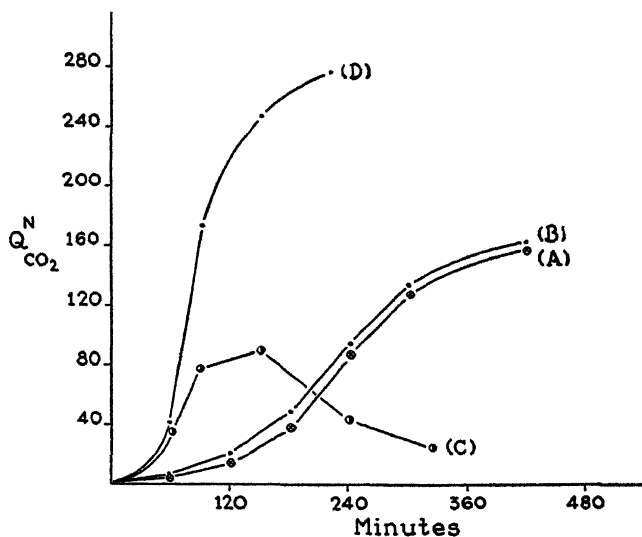
FIG. 4. Simultaneous adaptation to galactose and maltose. (B) is the curve obtained of galactozymase activity when the suspension is incubated in the presence of galactose only. (D) is the corresponding control curve for maltozymase activity. (A) and (C) are curves of galactozymase and maltozymase activities respectively when both are being induced simultaneously.

tose-fermenting capacity begins to make its appearance it can apparently drive the maltose-fermenting system out of existence. This latter finding was already apparent in the data reported in Table III. It is clear from curve (C) of Fig. 4 that while the presence of maltose does not prevent this effect of the galactozymase on the maltozymase it does slow the process of disappearance down. Thus, in the presence of maltose, galactose adaptation led to a fall from 90 to 43 in 90 minutes; i. e., a drop of 52 per cent. In the absence of maltose, in a comparable period under similar conditions the maltozymase experienced an 83 per cent decrease.

The effect of exogenous nitrogen on this type of simultaneous adaptation was also examined. The experiments were identical except that nitrogen was provided in the form of $(NH_4)_2SO_4$ in amount equivalent to 50 per cent of the

nitrogen content of the yeast. The results are recorded in Fig. 5. Curve (C) represents the galactozymase activity in the experimental suspension which was in contact with both substrates. The control for galactose adaptation is not reproduced since it does not differ significantly from the experimental. This is not surprising in view of the findings in the absence of exogenous nitro-
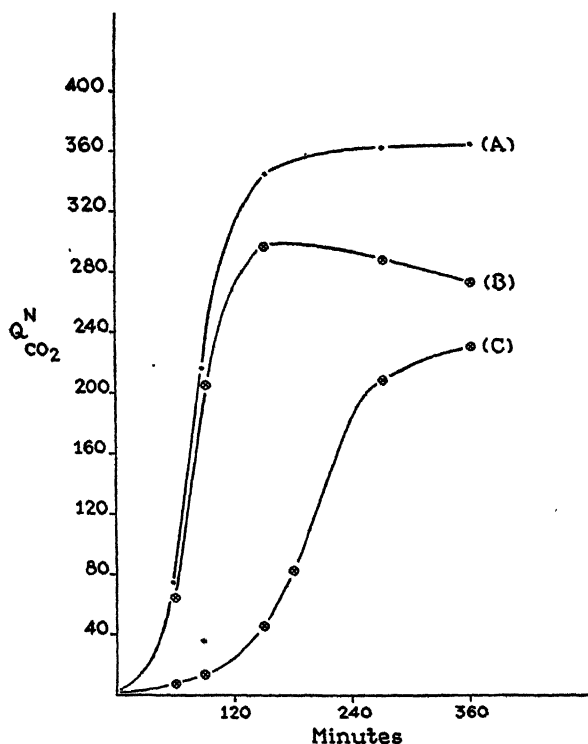
FIG. 5. Simultaneous adaptation to galactose and maltose in the presence of exogenous nitrogen. (A) and (C) are control curves of maltozymase and galactozymase respectively when each is induced separately. Curve (B) is the maltozymase activity attained in a cell in which galactozymase is simultaneously being induced.

gen. Curve (A) of Fig. 5 is the control curve for maltose adaptation in the presence of maltose only with added nitrogen. Curve (B) represents the maltozymase measurements on the experimental culture. Comparing curve (B) of Fig. 5 with curve (C) of Fig. 4 we see that the availability of an exogenous source of nitrogen has several striking effects. It raises considerably the attainable level of maltozymase in a culture which is simultaneously adapting to galactose. It also prevents considerably, although not completely, the destruction of the maltose-fermenting system which usually attends the appearance of

active galactose-fermenting capacity in the same cell. It is also worthy of note that the presence of both exogenous nitrogen and substrate greatly augments the capacities of each to protect the maltozymase system during galactose adaptation. As is seen from Table III nitrogen alone has only small protective action whereas substrate alone, as was noted above, confers protection to the extent that only a 52 per cent loss is observed in a 90 minute period. We note, however, from curve (B) of Fig. 5 that when both are present together even high galactozymase activities result in only a 7 to 8 per cent decrease in malto-zymase activity in 90 minutes.

TABLE IV

A Comparison of Fermentation Rates with Glucose, Galactose, and Maltose among Cultures Grown in the Presence of These Sugars

Each figure is the average of determinations on five different cultures, each of which was measured in duplicate. Average deviations from the mean are indicated.

Cultures grown in:	$Q_{CO_2}^N$	Strain	
		K	A
Glucose	With glucose	351 ± 8.5	249 ± 6.3
Galactose	With glucose	321 ± 9.1	232 ± 7.4
	With galactose	298 ± 18.2	224 ± 12.1
Maltose	With glucose	350 ± 7.5	·253 ± 8.3
	With maltose	343 ± 14.7	256 ± 12.1

(c) *Interactions between Adaptive and Constitutive Enzyme Systems*

The question arises whether the interaction effects observed between adaptive enzymes may not be peculiar to them as a class. It was therefore of some interest to see whether similar phenomenon did not exist involving some "constitutive" enzyme. The constitutive enzyme system chosen for examination was the glucozymase complex. In part the reasons for this choice can be seen in Table IV which records a comparison of the fermentation rates of glucose, galactose, and maltose amongst cultures grown in the presence of these sugars. It is seen that the activity of the glucozymase system is relatively unaffected by the particular carbohydrate used as the main source of carbon for the growth. It thus appears to satisfy the definition of a constitutive system. There are several other points of interest in this table. The $Q_{CO_2}^N$ values of a maltose-grown culture are the same whether glucose or maltose is being fermented and these values do not differ from that of a glucose-grown culture fermenting glucose. On the other hand, there is a significant though small (*ca.* 6 per cent) depression in the glucose-fermenting capacity as a result of growth in galactose.

It seemed not unlikely that this small effect of galactose adaptation on the glucozymase system could be magnified by carrying out the adaptation in the absence of exogenous nitrogen. Fig. 6 which examines glucozymase (B) and galactozymase (C) simultaneously in a suspension adapting to galactose in phosphate buffer confirms the supposition. Curve (A) is a non-adapting control testing the stability of glucozymase under the conditions of the experiment. The ability of exogenous nitrogen to abolish this effect is shown in Fig. 7. In
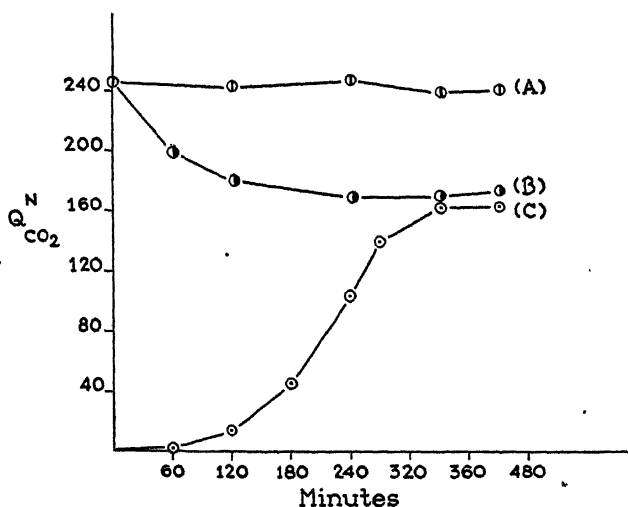
FIG. 6. The effect of adaptation to galactose on glucozymase activity. (A) is glucozymase activity of a non-adapting control; (B) is glycozymase activity of culture adapting to galactose. (C) describes the increasing galactozymase activity during the adaptation.

this experiment the same strain and experimental condition were employed except that exogenous nitrogen was available. It will be noted that no significant drop in the glucozymase system was observed during the period of the experiment. Apparently the 6 per cent decrease noted in Table IV requires relatively extended contact with galactose.

Though not as marked, these experiments would indicate that interactions between adaptive and constitutive enzymes do exist and can under the proper circumstances be exhibited. That not every enzymatic adaptation involving carbohydrate metabolism leads to loss in glucozymase was made clearly evident by experiments with maltose adaptation. Here a strain (K) was deliberately chosen with a high $Q_{CO_2}^N$ on glucose which exhibited some instability on removal of the glucose. This strain usually lost about 9 per cent of its activity when shaken in phosphate buffer in the absence of glucose. Fig. 8 describes an ex-
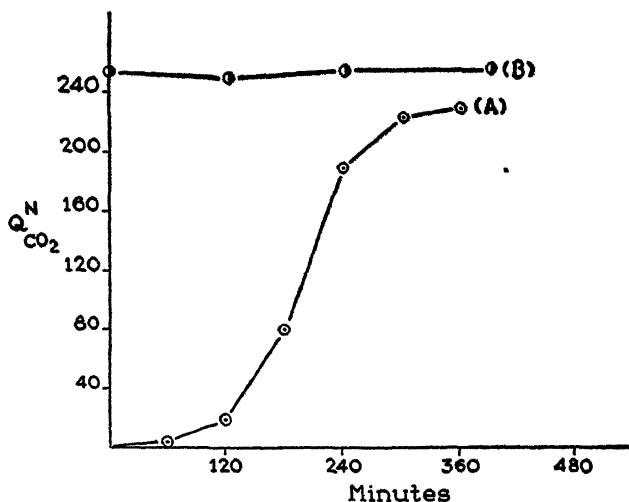
FIG. 7. The effect of exogenous nitrogen on interaction between galactozymase formation and glucozymase activity. (A) describes the increasing galactozymase activity; (B) describes the corresponding glucozymase activity.
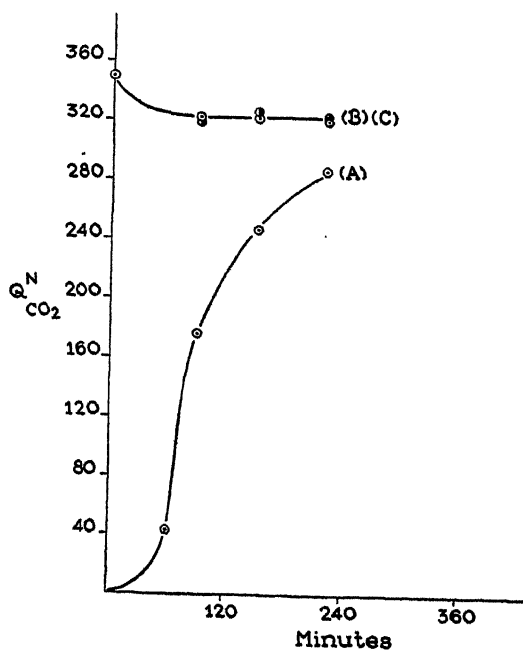
FIG. 8. The absence of effect of adaptation to maltose on glucozymase activity. (A) represents maltozymase activity; (B) the corresponding glucozymase values of the adapting culture; and (C) the glucozymase values of a control non-adapting culture.

periment in which strain K was adapted to maltose in the absence of exogenous nitrogen. Simultaneous maltozymase (A) and glucozymase (B) measurements were made. It is seen that adaptation to relatively high activity levels has no demonstrable effect on the course of events with the glucozymase system. The curve with the experimental culture (C) is indistinguishable from the control (C).

There exists no obvious relation between the severity of interaction and the amount of enzyme formed whether the interaction is between two adaptive enzymes or between an adaptive and a constitutive enzyme. This may be clearly seen in Table V which records increments in galactozymase for various intervals in the adaptation and the corresponding decrements experienced by the glucozymase system. During the first period an increase of 2.1 in galacto-

TABLE V

Comparison of Increments of Galactozymase Activity with Increments in Glucozymase Activity during Corresponding Intervals of Time during Adaptation to Galactose

Time interval	Galactozymase	Glucozymase
min.	$Q_{CO_2}^{N_2}$	$Q_{CO_2}^{N_2}$
0–60	2.1	45
60–120	10.9	20
120–180	32	5
180–240	58	5
240–360	59	0

zymase is paralleled by a 45 unit decrease in glucozymase. On the other hand during the interval in which the galactozymase has increased 59 units, no corresponding decrease is observed for the glucozymase system. Similar results are obtained if analogous calculations are made for two interacting adaptive enzymes.

IV

DISCUSSION

(a) *Relation of Adaptation to Nitrogen Metabolism*

The experiments reported here establish a connection between the adaptive process and the nitrogen metabolism of the cell. The ability of exogenous nitrogen markedly to stimulate the adaptation both in the rate of appearance and in the final amount of enzyme activity, would indicate that the adaptive process involves the formation of a nitrogenous compound.

It is of some interest to note that analogous findings have been reported for the glucozymase system in yeast by Winzler, Burk, and du Vigneaud (10).

These authors showed that biotin-deficient yeast had a low fermentation rate which could be greatly increased by the addition of both biotin and a source of exogenous nitrogen. Neither component when added alone was effective in raising the level of the fermentation rate. It was found that biotin-deficient yeast could not assimilate the added nitrogen unless some biotin was supplied. From these results it is clear that the mere presence of nitrogen *per se* in the medium is not sufficient. Assimilation is essential if the added nitrogen is to result in a stimulation of the fermentative process. This is in strict analogy with the experiments reported here, in which the addition of exogenous N had no effect on the rate or extent of adaptation unless active metabolism was occurring. Without the latter no assimilation of the nitrogen compound could occur. Winzler and his colleagues arrived at a conclusion similar to that offered above; *i. e.*, that the availability of an exogenous nitrogen source permitted the synthesis of a nitrogenous component necessary for fermentation.

The fact that a competitive interaction does exist between the enzymatic systems of a cell and the further fact that exogenous nitrogen can either abolish this interaction or modify its intensity constitutes strong evidence that the nitrogenous compound being formed during enzymatic adaptation is of protein nature. It further suggests that it is nitrogen which is being competed for when the enzymes within the cell interact during the formation of a new one. Independent evidence that a transformation of protein is involved was provided in studies (11) on the nature of adaptation to galactose fermentation. Separation of the galactozymase system into its apoenzymatic and coenzymatic components provided an opportunity for determining which had undergone a change in specificity as a result of the adaptation. The data showed unequivocally that it was the apoenzymatic or protein moiety which had been transformed. In view of these results it is not surprising to find competitive interactions between enzymes being formed in the same cell and that these interactions can be modified by providing a source of exogenous nitrogen.

It is at present impossible to say at what level the actual competitive interaction exists. That the nature of the interrelations between the enzymes of a cell is complex is apparent from the results obtained. It has already been emphasized that the amount of existent enzyme activity which disappears in a given interval bears no simple quantitative relation to the amount of new enzyme which is formed during the same interval of time. This fact would certainly rule out any hypothesis which assumes simple conversion of one enzyme molecule into another. It is not entirely unexpected to find that such is not the case. A simple one-to-one relation of this kind between the disappearance of one enzyme and the reappearance of another would only obtain under conditions where, *e. g.* two enzymes had the same prosthetic group which was being transferred from one to the other, or the same protein whose specificity was being modified in some way. The most probable situation to be ex-

pected would be one in which the formation of a particular enzyme would involve interactions with a whole series of enzymes the component parts of which are utilizable in its construction. Presumably certain enzymes would be preferentially used and, as these fall below certain critical values, others would be drawn upon to supply the material required for the formation of the enzyme being induced. When sufficient information becomes available, it should be possible to provide an ordered classification of the enzymes within a cell, based on the presence or absence and the extent of their interaction. This could well provide a type of enzyme classification of greater biological significance than that based on substrate specificity alone.

The severity of the interaction between two given enzyme systems would depend not only upon the similarity of their constitutions but also on the comparative ability of the systems which form them to compete with each other for material. Like any other physiological character, the synthesizing and competitive ability of a particular enzyme-forming system might well be expected to vary from one strain to another. It is therefore not surprising to find, as we do here in Table I, that in one strain of yeast (e. g., A) the presence of an active maltozymase system inhibits somewhat the activity of the galactozymase-forming system, whereas in another strain (e. g., K) no such effect is seen.

The extensive work of Monod (17) on the factors controlling enzymatic constitution in bacteria shows clearly that the same situation obtains in these organisms. This author was able to exhibit the successive appearance of enzymes in the cells of a culture exposed to two or more carbohydrates. A new enzyme system appears when the first substrate attacked becomes exhausted. The lag period between the exhaustion of the first substrate and the appearance of enzyme activity against the second is the basis for his *"diauxie"* phenomenon. From Monod's investigations it is evident that the type of enzyme interaction observed here in yeast is much more pronounced in the bacteria. Even with exogenous nitrogen present it amounts almost to a mutual exclusion effect. This is undoubtedly connected with the fact previously noted, that bacteria, with few exceptions, cannot adapt in the absence of exogenous nitrogen.

(b) "Adaptive" versus "Constitutive" Enzymes

It was of no little theoretical importance to find that interaction effects could also be observed with one of the so called "constitutive" enzymes. Karström (15) designated as "adaptive" those enzymes which are produced as a specific response to the presence of the homologous substrate. Such enzymes were differentiated from the "constitutive" ones which are always formed by the cells of a given species regardless of the presence or absence of their homologous substrates. It has already been pointed out (1, 2, 16) that it

seems doubtful whether classification into "adaptive" and "constitutive" enzymes is particularly useful or valid, implying as it does some sort of qualitative difference in origin and function between the two. Accumulation of data on enzymatic variation in microorganisms puts an ever increasing strain on the applicability of this kind of classification. Enzymes which have been labeled constitutive have been found to undergo wide fluctuations in the presence and absence of their substrate. Thus, the invertase content of *Bact. coli* rises to 452 (12) in the presence of sucrose and falls to values lying between 12.4 and 39 in its absence. Again, the *Q*-glucose values of *B. coli* are about 1,000 for organisms grown in the presence of glucose and about 190 for those grown in lactate medium (13). To these instances must now be added the findings reported in the present paper, in which it is shown that the so called constitutive enzymes can respond to changes in enzymatic constitution in much the same manner as the adaptive enzymes, although quantitative differences in the extent of the response certainly exist.

In connection with the general problem of classifying enzymes on the basis of their response to the presence or absence of their homologous substrates, there are certain important considerations which must be borne in mind. When we say that a given enzyme system, *e. g.* galactozymase, is purely "adaptive" we mean operationally that no galactozymase is found in the cell unless galactose is put into the medium. This is to be compared with the situation in the case of glucozymase, in which the addition of glucose as such to the medium is not necessary for the appearance of glucozymase activity of the cell. It is clear, however, that this distinction may lose much of its meaning if the cell can manufacture glucose from the non-glucose compounds that we add. Actually, all the biochemical evidence (14) indicates that this is precisely the condition that obtains in the case of galactose fermentation. Polysaccharides of glucose and intermediates of glucose metabolism have been found in cells which have been metabolizing galactose. Thus, even if glucozymase were a completely "adaptive" system, it would not be expected to disappear when the cell is metabolizing galactose, since the substrate of the glucozymase is always present. To conclude that glucozymase is constitutive (*i. e.*, its formation is independent of the presence or absence of substrate) because it is found when cells are metabolizing galactose, maltose, or some other carbohydrate would be misleading.

In general, whether an enzyme is classified as adaptive or constitutive may have little to do with any inherent properties of the enzyme itself. The critical factor may well be whether or not the cell can synthesize the homologous substrate. From this point of view it would be far more meaningful to classify substrates as constitutive or non-constitutive according as they are or are not synthesized by the cell. In operational terms, a particular enzyme could be classified as adaptive only if the experimenter is in a position to control the

presence or absence of the homologous substrate in his system. This obviously can be done only under one of two conditions. Either one must deal with substrates which the cell cannot synthesize (*e. g.*, galactose) or one must be able differentially to prevent the appearance of some substrate normally formed by the cell. The first of these two has been the one thus far employed in the study of adaptive enzymes. It is no mere accident that these studies have for the most part concerned themselves with enzymes not involved in catalyzing a step in the middle of a cyclic process. Enzymes so employed have their substrates made for them by the preceding step in the cycle. However, with enzymes like maltozymase or galactozymase, which *introduce* their substrates into a cyclic process, precise experimental control of concentration levels is possible.

(c) General Implications and Conclusions

The above analysis and the experimental facts presented lead to the conclusion that enzymatic adaptations are but quantitatively exaggerated instances of a more general phenomenon resulting from the effects of substrates on the synthesis and maintenance of their corresponding enzymes. The magnification in those cases which are classified as adaptive may well arise from the fact that in these instances one is dealing with substrates normally not found in the environment of the cell and which the cells are incapable of producing.

This viewpoint clearly does not deny the possibility of a classification of enzymes on the basis of stability. It does, however, raise certain fundamental questions on what is meant by stability. In addition to the problem of substrate still another aspect is pointedly emphasized by the experiments on enzymatic interactions. In view of the existence of such competitive interactions it is no longer *a priori* obvious that one is discussing the *chemical stability* of the *enzyme molecule* involved when a statement is made that a particular enzymatic activity disappears on removal of substrate. Enzymatic stability *in the cell* and its dependence on substrate can well be determined primarily by competitive interrelations between the protein-modifying systems which form the enzymes. The inherent chemical stability of the enzyme as an organic molecule may have little to do with the question. The latter characteristic can adequately be studied only when the enzyme has been isolated in relatively pure form.

We may make the problem more concrete by posing it in terms of a specific instance. The removal of galactose from the medium usually results in the disappearance of the galactozymase system. There are at least two alternative hypotheses which may be offered to explain this phenomenon.

1. The enzymatic component formed in response to the adition of galactose is a highly unstable protein molecule when uncombined with its substrate.

2. The protein molecule is stable but the enzyme-forming system involved

in its formation is a poor competitor for protein material in the absence of substrate. This results in the loss of the protein to other synthesizing systems.

The difference between these two hypotheses can most clearly be seen in terms of predator-prey relations encountered in the ecology of higher organisms. The prey may be a perfectly stable biological unit so long as it is not forced to coexist and compete in the same biological space as the predator.

It must be emphasized that these are not mutually exclusive alternatives since both mechanisms can and probably do function simultaneously in determining cellular enzymatic constitution. Experiments analyzing the relative importance of each are described in a subsequent paper (18).

The picture of the nature of enzymatic interplay under various conditions which emerges from the available data provides at least partial answers to some of the questions in the introductory paragraphs. The actual enzymatic constitution would appear to be in a state of continual flux. It can vary both by the loss of existent enzymes and the gain of new ones. There would appear to be little if any indifferent non-enzymatic storage protein in the cell which can be drawn upon for the formation of new enzymes. In the absence of an exogenous source of nitrogen, the formation of a new enzyme occurs at the expense of some of the existent enzymes. However, when an exogenous source of nitrogen is supplied this appears to be preferentially employed.

SUMMARY

Experiments on enzymatic adaptations in yeast to galactose and maltose under various conditions are examined. The pertinent facts established may be summarized as follows:—

1. The presence of exogenous nitrogen stimulates the rate of adaptation and raises considerably the attainable level of enzyme activity.

2. This stimulation is absent if the cells are unable to assimilate the added nitrogen.

3. Competitive interactions can be exhibited between two adaptive enzyme systems induced either serially or simultaneously in the same cell.

4. A similar kind of interaction was observed between an adaptive and a so called "constitutive" enzyme.

5. The presence of exogenous nitrogen modifies greatly the nature and extent of the interaction between the enzyme-forming systems.

The significance of these results to our understanding of the mechanism of the modification and maintenance of cellular enzymatic constitution is discussed. The validity of the distinction between "constitutive" and "adaptive" enzymes is reexamined in the light of the data presented.

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THE FORMATION AND STABILIZATION OF AN ADAPTIVE ENZYME IN THE ABSENCE OF ITS SUBSTRATE*

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I

INTRODUCTION

One of the most crucial aspects of enzymatic adaptation is the rôle of substrate in the induction and maintenance of enzyme activity. The experimental facts available are apparently unambiguous. The presence of substrate seems to be necessary for the appearance of the corresponding enzyme, and the removal of substrate leads to the disappearance of the enzyme it induced.

Most attempts to explain both the inducing and stabilizing effects of substrate on the so called "adaptive enzymes" have assumed that these enzymes form a class characterized by an unique *instability* in the absence of substrate. However, if such explanations are not to remain mere restatements of the experimental findings, a more precise concept of enzymatic stability must ultimately be provided. The need for such clarification is pointedly emphasized by the results of recent investigations in bacteria (1) and the yeasts (2), which have indicated the existence of competitive interactions among the enzyme-forming systems of the cell. The induction of a new enzyme in a cell can result in the disappearance of an existent enzyme and the suppression of others. In yeasts (2) the severity of this type of interaction can be reduced greatly by furnishing an exogenous nitrogen source.

Since the formation of a new enzyme can modify the activity levels of existent enzymes, enzymatic stability may be a more complex phenomenon than the more or less purely chemical concept which has thus far been considered. The existence of competitive interactions makes it pertinent to inquire whether the observed instability of the adaptive enzymes necessarily reflects their *chemical stability* as organic molecules.

It is evident that the maintenance of a particular enzyme in the cytoplasm can be determined by either one or both of the following factors: (1) The chemical stability of the enzyme as a protein molecule. (2) The competitive effectiveness of the synthesizing mechanism involved in its formation.

The effect of substrate in the induction and maintenance of a specific enzymatic activity could be mediated by either of these two factors.

It is the object of this paper to present experiments designed to assess the relative importance of these factors in the mechanism whereby substrates influence cellular enzymatic constitution.

The experimental procedures adopted depend primarily on arranging conditions which would tend to minimize or completely to abolish competitive interaction among the enzyme-forming systems of the cell. It was reasoned that, if the latter is a controlling element in the modification and maintenance of enzymatic constitution, then the reduction of such interactions should markedly increase the stability of the unstable adaptive enzymes even in the absence of their substrate. It might also be expected that the ability of substrate both to induce and maintain its enzyme would be augmented under such conditions.

Two general methods were employed to reduce the severity of competitive interactions among the enzyme-forming systems of the cell. One was to supply sufficient nitrogenous material and optimum conditions for their use, so that the requirements of all nitrogen-synthesizing systems could be satisfied. The second was to inhibit all enzyme formation so that no competitive interaction could occur. As will be seen, both of these methods resulted in marked stabilizations of enzyme content independent of the presence or absence of substrate.

All the experiments reported here were done with adaptation to galactose fermentation in yeast. This system was chosen because it represents one of the most carefully and fully investigated cases of enzymatic adaptation.

II

Methods and Materials

Two representatives of *S. cerevisiae* and one of *S. carlsbergensis* were used in the experiments reported. The strains employed, A1, K1, and C1, are the same as those used in previous investigations.

The details relating to the media employed, the methods for handling stock cultures, and the preparation of standard suspensions may be found in preceding (3) papers.

All enzyme activity measurements were performed at 30.2°C. with the standard Warburg apparatus. Anaerobiosis was established by replacing the air with nitrogen. Enzyme activities are expressed in terms of $Q_{CO_2}^{N_2}$ (cubic millimeters of CO_2 released anaerobically per milligram of dry weight per hour). In certain instances which are detailed in the text, it was more convenient to express activities in numbers proportional to $Q_{CO_2}^{N_2}$. Inaccuracies due to retention of CO_2 were reduced by using $M/15$ KH_2PO_4 as the suspending medium for the cells. Unless otherwise specified, measurement of enzymatic activity with respect to a given substrate was made with the latter at 3 per cent.

With the exception of modifications noted in succeeding sections, all adaptations were carried out with washed cells suspended in $M/15$ KH_2PO_4 in the presence of 4

per cent of the adapting substrate. These suspensions were shaken in flasks in a water bath held at 30.2°C. Adaptations under anaerobic conditions were carried out in large Warburg flasks, filled with nitrogen.

The density of the experimental suspensions was determined with the aid of a colorimeter (Klett-Summerson photoelectric colorimeter, filter 42), which was calibrated in terms of dry weight (milligrams per cubic centimeter of suspension).
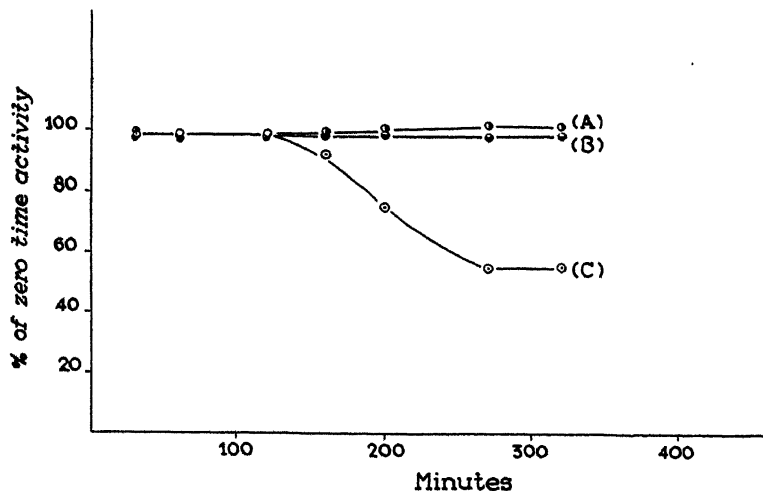
FIG. 1. The loss of adaptive galactozymase activity during the anaerobic fermentation of galactose (C). Illustrating the protective effect of exogenous nitrogen on the enzyme activity (A). Curve (B) shows the relatively greater stability of glucozymase even in the absence of nitrogen.

III

EXPERIMENTAL RESULTS

(a) *Factors Influencing the Capacity of Galactose to Stabilize Galactozymase*

There is no doubt that the removal of substrate often leads to the rapid disappearance of the corresponding enzyme. Nevertheless, the stabilization capacity of substrate is not an absolute one, and its presence does not guarantee the indefinite maintenance of activity. This can be seen clearly in some of the experiments reported by Stephenson and Yudkin (4), who showed that, even in the presence of galactose, the introduction of glucose can result in a marked diminution of galactozymase activity. However, the introduction of another substrate is not necessary to observe such losses. All strains of yeast examined, when fully adapted to galactose and allowed to ferment this hexose in phosphate buffer, will begin to lose activity after varying periods of time.

A case of this kind is exhibited in curve (C) of Fig. 1. This curve was ob-

tained by starting with a fully adapted culture of strain A, which was suspended in phosphate buffer containing 4 per cent galactose and allowed to ferment under anaerobic conditions. At intervals aliquots were removed, and the galactozymase and glucozymase activities determined. Even though this culture had been in continual contact with substrate, the galactozymase activity fell to 55 per cent of its initial activity. During the same period the glucozymase activity, which is given by curve (B), remained constant.

The loss of galactozymase during the metabolism of galactose is connected with the conversion of nitrogenous compounds. This is clear from curve (A) of Fig. 1, which describes the galactozymase activities of a suspension identical in origin with that employed for curve (C). In the case of curve (A), however, the fermentation of the galactose occurred in the presence of an exogenous source of nitrogen. The amount of nitrogen added (as $(NH_4)_2SO_4$) was equivalent to only 50 per cent of the nitrogen content of the cells. This amount of nitrogen is not sufficient to yield more than 5 per cent new cells under the conditions of the experiment. Nevertheless, complete stabilization of enzyme activity is observed. In the absence of substrate this amount of nitrogen is completely incapable of maintaining the galactozymase.

That relatively small amounts of nitrogen greatly augment the capacity of substrate to influence enzymatic constitution has already been seen (2) in the curves of enzyme *appearance*. Here, we see that the same amount of nitrogen can greatly augment the capacity of substrate to prevent the *disappearance of* the homologous enzyme. It seems likely, therefore, that we are dealing with the same phenomenon in these two instances; *i. e.*, the addition of an extra nitrogen source makes it easier for the galactozymase-forming system to make more enzyme.

These experiments do not permit a decision as to the primary mechanism underlying the disappearance of the adaptive enzyme in the presence of the substrate. They serve, however, to emphasize that conditions and factors other than substrate availability are of importance in determining enzyme activity levels. They further make unlikely any hypothesis of substrate function which depends solely on the capacity of substrate to stabilize the molecular structure of the enzyme. The marked ability of glucose to depress the stabilizing capacity of galactose and of ammonia to increase it are also not readily interpretable in such terms.

On the other hand, if competitive interactions are quantitatively important in determining enzymatic constitution, these results are not unexpected. The ability of substrate to stabilize its enzyme would be interpreted in terms of such factors as availability of nitrogen and the nature of other enzymes being used and formed by the cell.

(b) *Galactozymase in Resting Suspensions Metabolizing Various Substrates*

The competitive interaction interpretation of the inability of galactose completely to prevent galactozymase disappearance implies that enzyme turnover is taking place in a metabolizing cell. Under such conditions a slow loss of enzyme protein by the galactozymase-forming system to more active synthetic components could occur.

This viewpoint would predict that the capacity of a cell to retain galactozymase activity in the absence of exogenous nitrogen and galactose would depend on the level of metabolic activity and on the type of substrate being
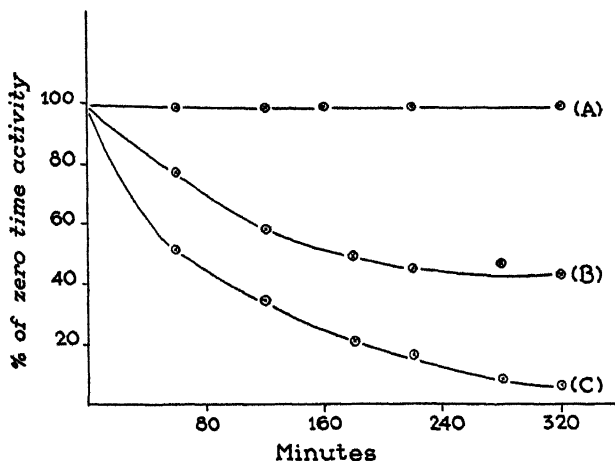
FIG. 2. The effect of endogenous respiration (B) and glucose utilization (C) on the stability of the adaptive galactozymase under aerobic conditions. The control curve (A), representing the results of aerobic incubation with galactose, illustrates the greater stability of the enzyme in this strain (K) as compared with the strain used in the experiments of Fig. 1.

metabolized. Presumably, the utilization of certain substrates could result in more severe interactions with the galactozymase system than others. It was therefore of some interest to see whether this was the case.

In order to magnify any differences in interaction effects, it was necessary to carry out these experiments with suspensions in phosphate buffer without any exogenous nitrogen. To provide a better basis of comparison, strains were employed here, which possessed a relatively better capacity to retain galactozymase activity than that exhibited by strain A in Fig. 1. Cultures fully adapted to galactose were washed free of medium and suspended in $M/15$ KH_2PO_4, to which was added the substrate to be metabolized. These suspensions were then incubated aerobically while shaking in a 30°C. bath. Aliquots

were taken at intervals, washed by centrifugation in the cold, resuspended in their original volumes of M/15 KH₂PO₄, and galactozymase activities determined.

The behavior of such suspensions is clearly illustrated in Fig. 2 which sets forth the galactozymase activities of strain K at various intervals of time while metabolizing galactose (curve (A)), endogenous reserves (curve (B)), and glucose (curve (C)). The relative stability of the galactozymase system in this strain metabolizing galactose in a nitrogen-free medium is illustrated by curve (A). It must, however, be noted that a fall in activity is observed if the incubation is carried out much longer. It is clear that the metabolism of the endogenous reserves leads to a considerable loss in galactozymase activity. The rate of decrease diminishes after about 120 minutes, and this may be connected with the fact that the rate of the endogenous respiration also begins to

TABLE I

Effect of the Metabolism of Various Substrates on Stability of Galactozymase

Activities are reported as microliters of CO_2 produced anaerobically from galactose per 30 minutes. The per cent entry gives the decrease in activity as per cent of zero time control. Respiration rate is given in terms of c. mm. of O_2 taken up per 30 minute period in the presence of the corresponding substrate at 0.01 M concentration.

Sample	Zero time	Galactose	Buffer	Glucose	Fructose	Alcohol	Pyruvate
Activity	474	429	316	149	147	261	265
Decrease, *per cent*	—	9	33	69	69	45	42
Respiration rate	—	191	87	200	179	256	131

drop sharply at this point. If the incubation is carried out for extensive periods of time (20 hours or more) the activity will, under these conditions, finally drop to about 5 per cent of its initial value. In the case where glucose is metabolized, a very rapid disappearance of the adaptive enzyme is observed. The reason for the more rapid decline of galactozymase activity in the suspension metabolizing glucose cannot be arrived at from these experiments. There exists here a difference in substrate as well as in metabolic rate, and either or both of these factors may be responsible. Nevertheless, it is evident that retention of the adaptive enzyme in the absence of substrate is dependent upon the metabolic activity of the cells.

Similar experiments in which these as well as other substrates were tested were carried out with strain C. Some typical results are recorded in Table I. For other than the zero time value, the numbers in the activity row in this table are those attained after 280 minutes of aerobic incubation at 30°C. in the presence of the corresponding substrate. The general pattern of behavior of the galactozymase with respect to the metabolism of galactose, endogenous reserves,

and glucose is the same as that observed with strain K. The metabolism of fructose appears to have a quantitative effect similar to that obtained with glucose. The consumption of both alcohol and pyruvate, however, results in a significantly smaller deterioration of galactozymase than that of the two hexoses. A comparison of the respiratory rates shows that this difference cannot be attributed to correspondingly lower metabolic rates for the alcohol and pyruvate. The former is actually metabolized at a higher rate than either hexose. Further, although alcohol is oxidized twice as fast as pyruvate, their efficiency in depressing galactozymase activity is virtually identical.

We have here a relatively clear cut case in which the type of substrate metabolized influences the stability of an adaptive enzyme in the absence of its substrate. This result is consistent with the hypothesis that competitive interactions are fundamental in the maintenance and modification of enzyme constitution. Aside from the general finding that different substrates vary in their effectiveness in suppressing the galactozymase activity, it is of some interest to note the order. Those which are most similar to galactose, *i. e.* glucose and fructose, are the most effective. This is the kind of order which would be expected if the intensity of competitive interaction is most pronounced between enzyme-forming systems controlling activity levels of enzymes handling the most closely related substrates.

(c) Stabilization of Galactozymase in the Absence of Substrate

The experiments described thus far show that the presence of substrate alone is not *sufficient* for the complete maintenance of the corresponding enzyme. It is the purpose of the present section to show that the presence of substrate is not *necessary* for enzyme maintenance.

In discussing the nature of adaptive enzyme formation and the rôle of substrate, it was pointed out that an assessment of the relative importance of competitive interactions could be attained by comparing enzyme stability under conditions where such interactions exist with those in which they have been suppressed.

Since effective competitive interaction presumably would occur only between actively synthesizing systems, suppression of interaction would be attained with the aid of any agent or condition which depressed enzyme formation to a considerable extent.

There are adequate experimental data available which demonstrate that the existence of an active metabolism is necessary for enzyme formation. It has been shown by various investigators (5, 6) that yeasts suspended under anaerobic conditions in the absence of an exogenous fermentable substrate metabolize at a negligible rate. Under similar conditions adaptive enzyme formation either does not occur at all or does so at a very low rate (7). These facts suggest that competitive interaction among enzyme-forming systems would

be sharply reduced in cells suspended in a substrate-free medium under anaerobic conditions. If such interactions do determine enzymatic stability to any considerable extent, an adaptive enzyme in the absence of substrate should be much more stable under anaerobic than under aerobic conditions, provided no other exogenous substrate is present.

A comparison of this kind between the aerobic and anaerobic stability of galactozymase is shown in Fig. 3. A galactose-adapted culture was washed and resuspended in M/15 KH$_2$PO$_4$. A portion of this suspension was shaken in air (curve (A)) at 30°C. and another shaken in nitrogen (curve (N)) at the same temperature. Samples were removed at intervals and galactozymase
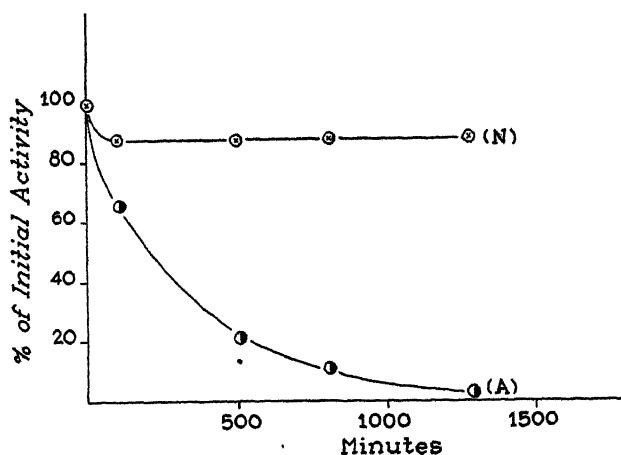
FIG. 3. The stabilization of adaptive galactozymase by anaerobiosis in the absence of substrate. The rapid disappearance of activity under aerobic conditions (curve (A)) contrasts with the marked stability appearing during incubation under N$_2$ (curve (N)).

activity levels determined. It is clear from a comparison of curves (N) and (A) that in the absence of active metabolism the presence of substrate is not necessary for the maintenance of the adaptive enzyme.

Another and perhaps more significant method of accomplishing a similar situation is provided by some recent experiments with inhibition of enzyme formation. It was shown (8) that the presence of NaN$_3$ in concentration of 2×10^{-3} M prevented the cell from utilizing the energy generated by anaerobic carbohydrate metabolism for adaptive enzyme formation. It should be recalled that this concentration of azide does not interfere with anaerobic fermentation of carbohydrate. In every instance the addition of the azide stopped any further enzyme formation, and the suspension maintained the activity attained at the time of the azide addition. If azide effectively blocks enzyme synthesis

in general, it can be expected, in view of the findings with anaerobiosis, that azide would also prevent the loss of any existent enzyme activity, no matter what substrate is being metabolized by the cell. Fig. 4 records the results of some experiments devised to test this expectation.

A fully adapted culture was washed and a suspension in $m/15$ KH_2PO_4 prepared in the usual manner. Galactozymase activity level was determined on an aliquot immediately. The remainder was dispensed in equal amounts into four flasks, into each of which was introduced an amount of glucose sufficient
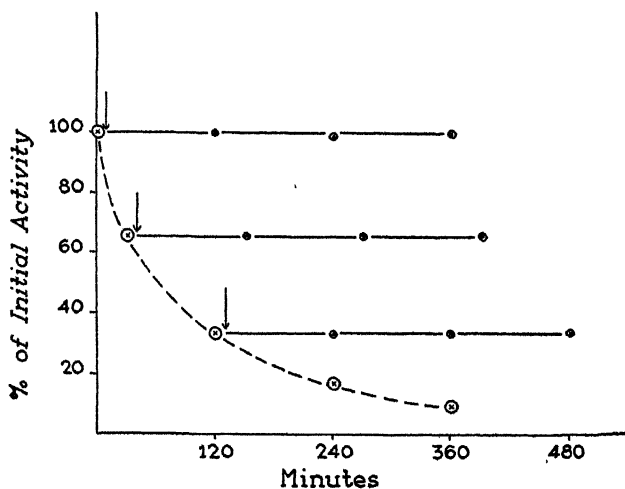
FIG. 4. The stabilization of adaptive galactozymase by NaN_3 during the anaerobic fermentation of glucose. The arrows indicate points where NaN_3 was added. The small circles (solid lines) show that NaN_3 prevented enzyme breakdown no matter at what level of enzyme activity the inhibitor was added. The large circles (dotted curve) show the loss of enzyme which occurs in the control suspension not treated with NaN_3.

to make a 4 per cent solution after anaerobiosis was established. In one flask azide (2.5×10^{-3} m final concentration) was put in immediately with the glucose. In a second, the azide was introduced after 30 minutes of glucose fermentation, by which time the galactozymase activity had fallen 34 per cent. The third flask received azide after 2 hours' anaerobic incubation with glucose. The fourth flask acted as an azide-free control. In each case, immediately before the introduction of the azide a sample was removed, washed in chilled buffer in the cold to remove the glucose, and galactozymase activity determination made. The large circles in Fig. 4 represent the galactozymase activity values attained by suspensions fermenting glucose in the absence of azide. The smaller circles show the behavior of galactozymase in cells fermenting

glucose subsequent to the addition of the azide. It is evident that in every instance the addition of azide stops any further enzyme disappearance, and the suspension maintains the activity attained at the time of the azide addition. A comparison of these results with those obtained with azide on anaerobic enzyme formation (8) reveals that the effect of azide on enzyme appearance is exactly similar to its effect on enzyme breakdown. In both cases azide can "freeze" the enzymatic constitution of the cell at that composition obtaining at the moment of its addition.
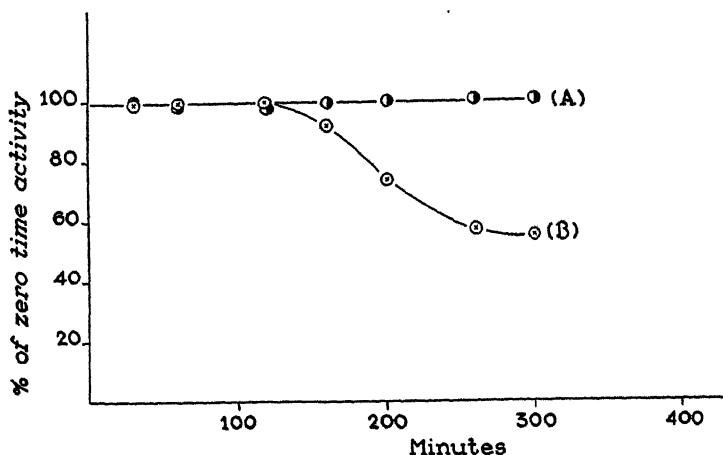
FIG. 5. The stabilization of adaptive galactozymase by NaN₃ during the anaerobic fermentation of galactose. The same conditions as those of Figs. 1 and 4. Curve (B) is a control, curve (A) shows the behavior in the presence of NaN₃. This brings out the essential similarity between the instability in the presence of the substrate and the instability in the presence of a competitive substrate.

A similar experiment performed in the presence of galactose instead of glucose (Fig. 5) shows that the loss of activity which occurs even in the presence of the adaptive substrate (see Fig. 1) can be prevented by azide just as can the loss in glucose, and that azide is able to replace exogenous nitrogen (Fig.1, curve (C)) in this respect. These results support the view that a common process of competitive interaction underlies the loss of enzyme activity under various conditions, and that the loss can be prevented equally well by stopping all competition or by removing the limiting factors which lead to competition.

(d) Formation and Maintenance of Galactozymase in the
Absence of Galactose

As was suggested in the introduction, the intensity of competitive interaction among enzyme-forming systems would in principle be decreased by

providing sufficient material to satisfy all their needs. Theoretically, one would suppose that this state of affairs would best be obtained under conditions in which growth is occurring. The very fact that growth is taking place implies that all or at least the essential protein-forming components are functioning effectively. If this be the case, it might be expected that the capacity of growing cells to maintain enzymes, unstabilized by substrate, would be observably greater than that of resting cells.

Experiments bearing on this point were performed with galactose-adapted cultures of strains K and C. 48 hour galactose-grown cultures were centrifuged, washed, and resuspended in glucose and galactose medium. In order to compare the enzymatic content of fast growing with that of slow growing suspensions, two different densities were made in the resuspensions. In one, called "heavy inoculation," the cells were resuspended in their original volume of medium. In the other, called "light inoculation," the density was adjusted to one-sixth that attained in the 48 hour culture. For purposes of comparison with results in glucose medium, a heavy type of inoculation was made into galactose medium. These suspensions were allowed to incubate, and samples were removed for galactozymase, glucozymase, and density determinations. As usual, enzymatic activity was assayed in terms of rate of anaerobic evolution of CO_2 in the presence of the corresponding substrate.

There were two measurements of primary interest. One was the total enzymatic activity, and the other was the enzymatic activity per cell. An estimate of the former could be obtained by measuring the activity per unit volume of the incubating suspension, and the latter could then be estimated from this measurement by correction for changes in cell density. In order to attain greater accuracy in the determination of enzyme activity, it was necessary to take large aliquots for measurement from the lightly inoculated suspensions, particularly in the case of the early samples. In all instances these samples were washed free of glucose and medium by centrifugation in the cold, and resuspended to adequate density in $M/15$ KH_2PO_4.

The results of these experiments are recorded in Table II. The density columns list milligrams dry weight of yeast cells per cubic centimeter. The total activity columns (4, 8) record the cubic millimeters of CO_2 evolved per hour per cubic centimeter. Division of these numbers by the corresponding density figures yields the values recorded in columns 2 and 6. These are therefore $Q_{CO_2}^{N_2}$ values since they represent cubic millimeters of CO_2 evolved per hour per milligram and in turn are proportional to activities per cell.

An examination of the density column shows that increases in the number of cells occurred in all cases. However, with both strains the lightly inoculated suspension showed over an eightfold increase as compared with slightly more than twofold for the heavy inoculation. The behavior of galactozymase activity during the course of this experiment can most readily be seen from Fig. 6,

TABLE II

Formation of Galactozymase in the Absence of Galactose during Growth

Strain	Treatment	Time	Density	Glucozymase					Galactozymase			
				(1) Ratio to zero time	(2) Activity/cell	(3) Ratio to zero time	(4) Activity/cc.	(5) Ratio to zero time	(6) Activity/cell	(7) Ratio to zero time	(8) Activity/cc.	(9) Ratio to zero time
		hrs.										
KG	Heavy inocula-	0	1.8	1.0	319	1.0	574	1.0	231	1.0	416	1.00
	tion into glu-	5	2.6	1.4	808	2.5	2100	3.7	127	0.55	430	1.03
	cose medium	21	4.2	2.3	584	1.8	2455	4.3	103	0.45	435	1.04
	Light inoculation	0	0.3	1.0	319	1.0	96	1.0	231	1.00	69	1.00
	into glucose	5	1.0	3.3	660	2.1	660	6.9	79	0.34	79	1.15
	medium	21	2.6	8.6	629	1.9	1630	17.0	51	0.22	132	1.92
	Heavy inocula-	0	1.8	1.0	319	1.0	574	1.0	231	1.00	416	1.00
	tion into galac-	5	2.4	1.3	637	2.0	1530	2.7	477	2.07	1145	2.75
	tose medium	21	4.5	2.5	422	1.3	1900	3.3	304	1.31	1369	3.30
CG	Heavy inocula-	0	1.7	1.0	418	1.0	711	1.0	185	1.00	315	1.00
	tion into glu-	6	3.7	2.2	654	1.6	2420	3.4	85	0.46	315	1.00
	cose medium	26	3.8	2.2	453	1.1	1700	2.4	93	0.50	349	1.11
	Light inoculation	0	0.28	1.0	418	1.0	118	1.0	185	1.00	53	1.00
	into glucose	6	0.90	3.8	689	1.6	620	5.2	90	0.49	82	1.55
	medium	26	2.30	8.2	616	1.5	1400	11.9	35	0.19	98	1.85
	Heavy inocula-	0	1.7	1.0	418	1.0	711	1.0	185	1.00	315	1.00
	tion into galac-	6	3.2	1.9	450	1.1	1440	2.0	376	4.93	1205	3.83
	tose medium	26	4.3	2.5	380	0.9	1615	2.3	138	0.75	586	1.86

The data were obtained by measuring anaerobic CO_2 production in the presence of glucose or galactose by aliquots of cultures, treated as described in the first column. Columns (3) and (8) are the activities of equal aliquots of the experimental cultures; they show the course of the total enzyme activity of each culture during its growth. Columns (2) and (6) were obtained from columns (3) and (8) by correcting the values for the progressive change in the quantity of yeast resulting from growth. This is given by the density column, which is expressed as milligrams dry weight of yeast per cubic centimeter of culture. The values in (2) and (6) are therefore proportional to the $Q_{CO_2}^{N_2}$ and to the activity per cell. Each of the ratio columns (1), (3), (5), (7), and (9) gives the ratios of the values in the preceding column to those at zero time. They permit a comparison on a relative basis of the changes occurring with time under various conditions in the two enzyme systems examined.

in which are plotted the per cent of initial activity, both total and per cell, for both types of suspensions in the case of strain K. Included also is a curve obtained for the same strain fermenting glucose in the complete absence of growth.

It is seen that, for both the heavy and lightly inoculated suspensions, the enzyme activity per cell drops markedly. The decrease is more drastic in the case of the more rapidly dividing culture. However, the total activity remained absolutely constant in the case of the heavily inoculated suspension, and increased almost twofold in the more rapidly dividing suspension. This
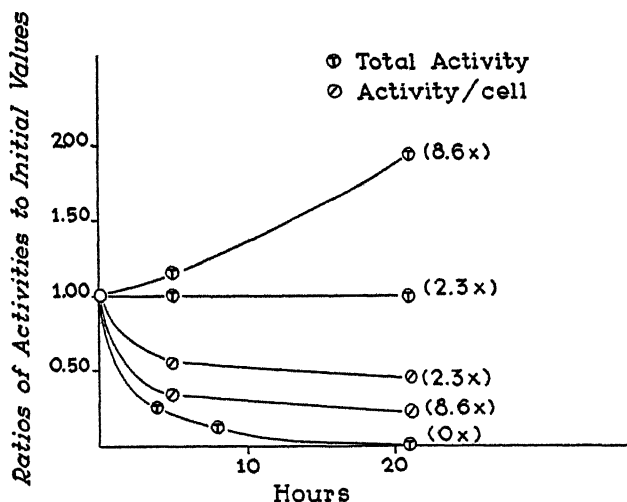
FIG. 6. The formation and maintenance of adaptive galactozymase by growing cells in the absence of galactose. Glucose was the carbohydrate source, and growth occurred in a complete medium (yeast extract-peptone broth). The designations (2.3 ×) and (8.6 ×) give the factor by which the initial number of cells increased during the experiment. The lowest curve shows the fall of enzyme activity in the absence of growth (medium: buffer plus glucose). The next two curves show the loss of activity per cell during increase of cell number; the more rapidly growing culture showed the most loss. The two uppermost curves show the total activity of the culture. They demonstrate the ability to maintain (slowly growing) and even to increase (rapidly growing) the initial enzyme activity in the absence of substrate under favorable conditions.

is to be compared with the total activity curve of the non-dividing suspension. In this latter case, rapid disappearance of the galactozymase occurs.

We have here a rather remarkable instance of adaptive enzyme formation in the absence of its substrate. The fact that an increase in galactozymase activity occurred in the more rapidly dividing suspension, whereas it was only maintained in the more slowly growing culture, is not unexpected. Presumably, the closer the approximation to optimal conditions of growth, the less severe are the competitive restrictions, resulting therefore in a more favorable condition for the synthesis of unstabilized enzymes.

Examination of Table II shows that an exactly similar situation obtained in the case of strain C. Here again, the rapidly dividing suspension exhibited the capacity to form galactozymase in the absence of galactose, and the more slowly growing suspension maintained its activity. As in the case of strain K, the activity per cell decreased in both suspensions, and again it is seen that the more rapidly growing culture exhibits a greater drop in activity per cell. This is probably due to the fact that the rate of enzyme formation is not rapid enough to keep up with the rate of production of new cells. Thus, as time went on, the new cells received less and less enzyme.

As an example of the possibility of rather extensive variations in the content of a so called constitutive enzyme, it is of some interest to examine column 2 of Table II. This records the glucozymase activity obtained during the course of the incubation. It will be noted that the glucozymase activity per cell rose to a peak at the 5 hour period and then fell. The same situation is observed for the galactozymase activity of the heavily inoculated suspension in the galactose medium (column 6).

DISCUSSION

The data presented permit a discussion of some of the fundamental aspects of enzymatic adaptation and a comparison of the adequacy of various hypotheses which have been offered in an attempt to explain this phenomenon. We need not concern ourselves here with those explanations which are based on a supposed "activation" of a preexisting enzyme system. These have already been discussed fully in a previous paper (3). It may merely be pointed out here that such theories are made quite untenable by the finding (9) that enzymatic adaptation in the case of galactose involves a modification of the apoenzymatic or protein moiety of the enzyme system. In addition, they either ignore or fail to explain the rôle of the substrate in the adaptive processes.

In the main, there have been proposed three types of theories which provide a reasonable mechanism whereby a cell can modify its enzymatic constitution in response to substrate. We may summarize these in the following way:

1. The Mass Action Theory.—Yudkin (10) assumed that the presence of substrate stimulates the production of its homologous enzyme by shifting an equilibrium between the enzyme and its precursor in favor of more enzyme formation. The underlying assumption is that the combination of enzyme and substrate, by removing the free enzyme from the reaction system, leads to further enzyme production.

2. Pre-Enzyme Theory.—The pre-enzyme theory of Monod (11) assumes the existence of a pool of common precursor from which a number of different enzymes can be formed. This precursor or pre-enzyme is supposed to have a small but finite affinity for any of the substrates. The combination of one of

these substrates with the pre-enzyme changes it, presumably by conferring a definite specificity upon it, in such a way as to form the particular enzyme.

3. *The Plasmagene Theory.*—This hypothesis asserts (12) that each enzyme is produced from non-enzymatic material by a specific enzyme-forming system, the plasmagene, which is self-duplicating. It is assumed that substrate, by combining with enzyme, increases the stability of the plasmagene-enzyme complex. Since the unit stabilized is autosynthetic, an increased net rate of enzyme formation would necessarily result.

(*a*) *The Mass Action Hypothesis.*—The adequacy of the mass action theory, as formulated by Yudkin, has previously been criticized on kinetic grounds (12–14). The mass action theory predicts that the adaptive curve should always be concave to the time axis. The observed rate of increase in enzyme activity should be maximal at the onset and decrease continuously until full activity is reached. A careful analysis of the kinetics of adaptation in all instances in which adequate data are available reveals that, on the contrary, the early portion of the adaptive curve is exponentially increasing in character.

Any theory of enzymatic adaptation which proposes to explain the rôle of substrate in inducing the appearance of enzymatic activity must also provide an explanation of why the enzyme disappears on the removal of the substrate. The mass action hypothesis provides a perfectly clear cut and testable mechanism for the stabilizing capacity of substrate. It implies that the reaction between precursor and enzyme has its equilibrium point far over to the side of the precursor. The mechanism suggested to explain the disappearance of enzyme on removal of substrate is therefore the essential instability of the enzyme molecule when it is uncombined with its substrate.

Such a hypothesis would obviously fail to explain some of the facts reported in the present paper. It would be difficult to predict from this hypothesis, for example, that the utilization of some other substrate in the presence of the adaptive one should lead to a decrease in activity of the adaptive enzyme.

It would also be difficult to predict the possibility of stabilizing enzymes in the absence of their substrates by the very conditions which inhibit or prevent enzyme formation. The fact that both anaerobiosis and the presence of sodium azide lead to maintenance of galactozymase activity, even in the absence of galactose, almost directly contradicts one of the primary postulates of the mass action hypothesis: for it shows that the adaptive enzyme is *not an inherently unstable unit in the absence of the substrate.*

Finally, the rôle assigned to substrate in this theory is one that would not permit measurable amounts of enzyme to be formed in the absence of substrate. Yet, as we have seen, under appropriate conditions precisely this result can be obtained.

(*b*) *The Pre-Enzyme Hypothesis.*—Monod formulated the pre-enzyme hypothesis largely in order to account for a discrepancy between the mass

action theory of Yudkin and Monod's own results (11) on the effect of substrate concentration on rate of adaptation. According to the mass action theory, substrate concentrations greater than that required to saturate the number of enzyme molecules present should have no effect upon the precursor-enzyme equilibrium, and therefore should have no effect upon the rate of enzyme formation. Monod, studying adaptive maltozymase formation in *E. coli*, observed a marked effect of substrate concentration above the concentration which he finds to be the saturation value for fully adapted cells. He argues from this that substrate must have another rôle than that of combining with and stabilizing the enzyme. Accordingly, he assigns substrate the function of combining with the precursor or pre-enzyme and converting it to enzyme. Since the quantity of hypothetical pre-enzyme may be very large, and its combining capacity low, it is possible to understand how more substrate would be required to saturate it than is required to saturate the maximal amount of enzyme formed.

Monod's theory was thus primarily devised to explain the effect of substrate concentration on enzyme formation. Accordingly, it does not specifically discuss the reasons why an enzyme, once formed from pre-enzyme by substrate, disappears when the substrate is removed from the medium. By implication it imputes a greater degree of instability to the enzyme in the absence of substrate.

The pre-enzyme theory does imply that competition occurs during enzyme formation—competition of substrate for the pre-enzyme. However, it does not provide for competitive interactions which are not directly mediated by substrate. It is therefore difficult to explain in terms of this hypothesis some of the experiments reported here on enzyme disappearance in the presence of substrate.

A crucial test of the applicability of Monod's theory must of necessity center around the rôle assigned to substrate in enzyme formation. One must conclude from the primary postulate of his proposed mechanism that the presence of substrate is *necessary* for enzyme formation. It is clear that this conclusion, and therefore the postulate from which it is derived, is untenable. The data presented above on rapidly growing cells demonstrate that adaptive enzyme can be formed in the absence of the corresponding substrate. One must conclude from these experiments that the presence of substrate is a *sufficient* rather than a *necessary* condition for enzyme formation.

It must be emphasized that none of the experiments described here rule out the existence of a pre-enzyme in the sense of an immediate enzyme precursor already possessing some specificity towards substrate. Neither do these results disprove the possibility that substrate can aid in the conversion of pre-enzyme to enzyme. They do prove that this conversion can occur without the intervention of substrate.

(c) *The Plasmagene Theory.*—The plasmagene theory differs from the other

theories of enzyme formation in that it does not assign the primary part in determining enzyme formation to substrate. The assumption that independent enzyme-forming units exist and that they are self-duplicating permits the utilization of what is already known concerning competitive relations between systems of self-duplicating units. Within this framework substrate provides *one* of the factors which can determine the successful competition of one particular system of self-duplicating units. Other factors known to operate are availability of energy, availability of nitrogenous building materials, and the general metabolic rate of the cell. The evidence for competitive interaction in enzyme formation, together with the effects of these factors, has already been discussed elsewhere (2).

The plasmagene theory does not assume any inherent instability of the molecules of active enzymes. It is clear that if raw materials or energy, or both, are limiting factors in enzyme formation, one would expect that some enzymes would be built up at the expense of others—the more able competitors at the expense of the less able. If combination of plasmagene and enzyme with substrate increases the stability of the complex, it would be expected that the presence of substrate would promote the appearance of measurable quantities of enzyme under conditions where otherwise no such quantities would survive the competitive process.

This point of view implies that the presence of substrate by itself does not guarantee the stability of enzyme once it has been formed. If cells are placed in a medium containing no external nitrogen source, and various plasmagene systems are striving to build enzyme, one would anticipate a loss of adaptive enzyme even in the presence of substrate. This is precisely what we have found to be the case in the experiments reported in the first section of this paper. Moreover, one would predict that the addition of a supply of nitrogen sufficient for synthetic purposes should ameliorate the competition, and possibly abolish the interaction altogether. This also was found to be the case.

It obviously also follows from the theory that the competitors of a particular enzyme system should be more effective in wiping it out when placed in the presence of their own specific substrates. The experiments of the second section fulfill this prediction. They also indicate a certain order of enzyme-forming systems with respect to the intensity of competition between them. Thus, several substrates increase the breakdown of galactozymase above the rate found in buffer. But also, the hexoses glucose and fructose appeared to compete more keenly with the galactose system than alcohol or pyruvate. There is a suggestion in this that enzymes which act on chemically similar compounds (so that one would expect the enzymes to be structurally similar) are more easily interconvertible. This would be expected to lead to stronger competition between such systems than between systems dealing with chemically rather different substrates.

On the view proposed here, when an enzyme disappears it is essentially

because some other enzyme is actively being constructed, not because it spontaneously disintegrates. Therefore, anything which prevents enzyme formation should prevent enzyme breakdown equally well. The experiments of our third section show that this is the case. Enzyme-forming processes can be blocked by instituting anaerobic conditions in the absence of any fermentable material. They can also be blocked by the use of sodium azide. These conditions presumably stop synthesis by interfering with the production or the transfer of the energy required for synthesis. That they do prevent enzyme synthesis has already been amply demonstrated. In the experiments reported above, it is seen that they also effectively prevent the disappearance of an adaptive enzyme in the absence of its substrate.

According to the plasmagene theory, enzyme formation is not a simple response to the stimulus of added substrate. Plasmagenes are units which form their corresponding enzymes whenever favorable conditions for synthesis and self-duplication are provided. This would lead us to expect that, beginning with an adequate number of plasmagenes, enzyme could be formed in the complete absence of substrate, provided the supply of energy, nitrogen, and all other requirements for synthetic activity were favorable. The experiments with rapidly growing cultures in the fourth section of this paper provide this kind of situation. There is a low population density, an ample supply of energy, of nitrogen in various forms, and of growth factors. Accordingly, we find that in the presence of glucose and the absence of galactose—two conditions which would be expected to lead to a rapid decrease of galactozymase— the initial amount of galactozymase in the culture was actually doubled. Even a more slowly growing culture under these conditions was able to maintain its initial amount of adaptive enzyme intact, whereas the same culture, under conditions less favorable to synthesis, lost it very rapidly.

The experiments reported and discussed in this paper constitute a critical test for any theory of enzyme formation. If we assume that enzymatic constitution is determined by competitive interactions between self-duplicating enzyme-forming systems, we arrive at the simplest interpretation so far suggested which is consistent with all the facts known about enzymatic adaptation. The effects of various agents and conditions on the ability of cells to form new enzymes and maintain existent ones can then be understood in terms of their influence on the outcome of this competitive interaction.

SUMMARY

The effect of various conditions on the stability of adaptive enzymes in the absence of their substrates has been studied. In particular, it is shown that even the presence of substrate is unable to maintain adaptive enzyme indefinitely under conditions unfavorable to synthesis. The stability of the enzyme can, however, be insured by providing an exogenous nitrogen source.

It is shown that various substrates accelerate the disappearance of an adaptive enzyme when its own substrate has been removed from the medium. The order of effectiveness of such substrates appears to be connected with their chemical similarity to the adaptive substrate.

It is shown that two conditions which are able to inhibit the formation of adaptive enzymes—anaerobiosis and the presence of sodium azide—are equally able to prevent the disappearance of an adaptive enzyme after the removal of its substrate. Finally, it is shown that rapidly growing cultures, under optimal conditions for synthetic activity, are able to maintain and even appreciably to increase their initial content of an adaptive enzyme, in the absence of its specific substrate and in the presence of a normally competitive substrate.

In the light of these results, the three major theories of enzyme formation hitherto proposed are evaluated.

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STUDIES ON A NEW METABOLITE AND ITS OXIDATION IN THE PRESENCE OF ASCORBIC ACID*

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Kohn and Liversedge (1) have shown that tissues incubated aerobically produce a substance which reacts with p-aminobenzoic acid (PAB) to form a yellow soluble compound and with thiobarbituric acid to form a red soluble complex. Brain produces the greatest amount per unit weight, followed by liver and kidney, and traces were found in other tissues. The substance is produced in broken-cell suspensions or in tissue slices, but only in the presence of oxygen. A number of drugs inhibit the reaction and the authors postulated that an enzyme oxidizes a precursor present in these tissues to a product which would then react with either p-aminobenzoic acid or thiobarbituric acid. It seemed likely that this product was an aromatic aldehyde with hydroxy groups attached to the ring because p-hydroxybenzaldehyde, but not benzaldehyde, condenses with p-aminobenzoic acid to produce a yellow color, the absorption spectrum of which is similar but not identical with that given by the oxidation product of the tissues. Because of the possible importance of this oxidation, it was of interest to study it in greater detail.

EXPERIMENTAL

Rat brains were used for most of the experiments. They were ground in a mortar and squeezed through muslin. Approximately 90 mg. wet weight of tissue suspended in 0.5 ml. M/20 Na-K phosphate buffer pH 6.7 were incubated in flasks with 1.5 ml. of buffer and shaken in air at 37–38° C. for varying periods of time. At the end of the incubation period 1.0 ml. of 20 per cent trichloroacetic acid and 1.0 ml. of 1 per cent p-aminobenzoic acid were added to 2.0 ml. of suspension and the precipitate was centrifuged down. The liquid was poured off, diluted to 10.0 ml. with water, and the color read in an Evelyn colorimeter with a 420 filter. The readings obtained were proportional to the concentration at low concentrations only. A calibration curve was used to make necessary corrections at higher concentrations.

The first experiments were based on the assumption that an enzyme present in brain was oxidizing a substrate. Therefore brain suspensions were thoroughly washed five times with excess buffer pH 6.7 by centrifugation. Such

suspensions when incubated gave little or no color with PAB, presumably because the substrate had been removed by the washing. In order to obtain a concentrated solution of substrate brains were ground in 20 per cent trichloroacetic acid, extracted for 30 minutes, centrifuged, and the liquid extracted with washed ether to remove the trichloroacetic acid. The solution was then adjusted to pH 6.7. On incubation it produced no color with PAB but the addition of washed brain produced a color, the intensity of which, however, was not proportional to the amount of substrate solution. This fact was not consistent with a simple enzyme-substrate relationship.

The properties of the washed brain suspension were then investigated. It was placed in a boiling water bath for varying lengths of time and subsequently incubated with the "substrate" solution. Boiling for 10 minutes, which completely inactivated the enzymes responsible for the oxidation of glucose, lactate, pyruvate, tyramine, and p-phenylenediamine, only slightly impaired the color production with PAB. Even after 60 minutes boiling some activity remained. The enzyme, if such it were, was very thermostable. The washed brain could be precipitated with alcohol and acetone and kept for several months in the dry state without loss of activity. Treatment with strong acid, however, inactivated it.

Boiling the "substrate" solution in acid and alkali caused partial inactivation. Addition of small amounts of H_2O_2 for 10 to 15 minutes before incubation with washed heated brain cause complete inactivation. Removal of the excess H_2O_2 with blood catalase before incubation gave the same result, indicating that the "substrate" was inactivated by H_2O_2 and not the washed brain.

The reaction between the washed heated brain and the "substrate" solution can be inhibited by a number of compounds. The most active of these inhibitors are the aromatic hydroxy compounds such as epinephrine, catechol, quinone, and gallic acid. This effect along with certain others is shown in Table I. Metal ions, with the exception of copper which inhibits, are without effect in the concentrations used. Rat and rabbit serum also inhibit, possibly by adsorbing the "substrate." Washed heated brain and "substrate" solution when mixed take up oxygen and this uptake is also inhibited by the same compounds which inhibit the production of the substance that condenses with PAB. These compounds added after the incubation but before the addition of PAB have no effect; $i.e.$, they do not interfere with the condensation.

The occurrence of the reaction in tissues of various animals is shown in Table II. The tissues were ground but not washed and incubated at pH 6.7. The results are expressed as units per gram wet weight of tissue. In certain instances "substrate" solution from rat brain was added because of the probability of the "enzyme" being present but the "substrate" lacking. In no case did the addition of "substrate" to a negative tissue change the result. Apparently the two factors, if present, are present together.

Other than the production of the substance which condenses with PAB and the oxygen uptake there was no clue as to the nature of the reaction. No ammonia was produced, no –SH groups appeared or disappeared, and a number of spot tests for various groups were negative. It was found, however, that the addition of small amounts of ascorbic acid to washed heated brain in the absence of "substrate" caused the production of the substance which condenses

TABLE I

The Effect of Various Substances on the Washed Heated Brain Preparation in the Presence of "Substrate" Solution or Ascorbic Acid

Several different brain preparations were used. Incubation 45 minutes in air at 37°C. The substances added at the end of the incubation period before the PAB do not inhibit the color formation. The figures are in arbitrary units. The inhibitor concentrations are in milligrams per 2.0 ml.

Brain preparation + "substrate"	Brain preparation + "substrate" + inhibitor	Percentage inhibition	Brain preparation + ascorbic acid	Brain preparation + ascorbic acid + inhibitor	Percentage inhibition	Inhibitor
345	80	77	527	168	68	0.5 mg. epinephrine
424	401	5				0.5 mg. ephedrine
350	54	84	549	178	68	0.5 mg. catechol
316	77	76	549	326	41	0.5 mg. gallic acid
350	227	35	549	485	12	1.0 mg. resorcinol
308	44	86	549	165	70	1.0 mg. *p*-cresol
308	257	17	549	435	21	1.0 mg. tyramine
308	308	0	549	549	0	1.0 mg. tyrosine
424	345	19	549	527	4	0.1 mg. NaCN
175	146	17	549	390	29	1.0 mg. thiourea
286	54	86	520	69	87	0.5 mg. $CuSO_4$
308	96	68				1.0 mg. alloxan
331	56	83				0.2 mg. hydroquinone
385	110	73	596	140	77	0.1–0.3 ml. rat or rabbit serum
385	274	29				10 mg. purified globulin

with PAB. The absorption curve of the color thus produced was identical with that produced when the "substrate" solution was used, both being identical with the curve found by Kohn and Liversedge. Apparently ascorbic acid in the "substrate" solutions was responsible for the reaction. Further evidence for the identity of ascorbic acid and the "substrate" is shown in Table I. The same compounds which inhibit the reaction with the "substrate" inhibit the reaction when ascorbic acid is used. Moreover, pretreatment of ascorbic acid with H_2O_2 inactivates it just as it inactivates the "substrate."

A more quantitative study of the reaction was now possible because pure

ascorbic acid instead of "substrate" could be used. It was found that the amount of color produced when ascorbic acid was incubated with the washed heated brain preparation was independent of the ascorbic acid concentration down to 50γ. Below this amount rough proportionality was obtained. On the other hand the color was proportional to the amount of brain preparation.

TABLE II

The Activity of Tissues of Various Animals Incubated in the Presence of Either "Substrate" Solution or Ascorbic Acid

The figures are in arbitrary units and represent the amount of color produced per gram of wet weight of tissue after 45 to 60 minutes' incubation in air at 37°.

Animal	Tissue	Colorimeter reading	Animal	Tissue	Colorimeter reading
Frog					
Rana pipiens	Brain	2578	Rat	Brain	5920
	Gastrocnemius muscle	0, 336, 1790		Kidney	803
				Liver	1030
	Gastrointestinal tract	660	Guinea pig	Kidney	568
	Heart	111			
	Liver	0		Liver	518
	Kidney	0	Mouse	Brain	1220
				Kidney	819
Turtle				Liver	375
Pseudemys troostii	Brain	1176	Rabbit	Brain	394
	Striated muscle	0		Kidney	48
	Small intestine	35		Liver	24
	Heart	0		Heart	0
	Kidney	23		Adrenals	73
	Pancreas	30			
Molluscs					
Macrocallista nimbosa	Mantle edge	110			
	Gill	418			
	Adductor muscle	105			
Venus mercenaria	Mantle edge	426			
	Gill	281			
	Adductor muscle	117			
Ostrea virginica	Mantle	274			
	Adductor muscle	185			
Busycon carica	Foot	25			

Experiments on the oxygen uptake showed the same relationship. Table III and Fig. 1 show these results. Presumably, therefore, ascorbic acid catalyzes the oxidation of a compound which is linked to the brain protein and is not removed from it by washing with water, alcohol, or acetone. During the oxidation the linkage is broken and the compound goes into solution and can then react with PAB.

The ascorbic acid was estimated before and after incubation with the brain preparation by indophenol titration and by the method of Roe and Kuether (2). The values by the first method were as follows: with 0.5-ml. of brain preparation to which 0.5 and 0.1 mg. of ascorbic acid had been added: before incubation, 4.7 and 1.2 ml.; after incubation, 3.3 and 0.2 ml. respectively. The ascorbic acid is therefore oxidized in the process. The PAB color values for the two concentrations of ascorbic acid were 564 and 520. The Roe and Kuether method which estimates all the ascorbic acid as dehydroascorbic acid gave a colorimetric value of 265 before and after incubation of 0.1 mg. of ascorbic acid with the brain preparation. The value obtained on 0.1 mg. of ascorbic acid alone was 265 and brain alone gave 19. Therefore almost all the ascorbic acid incubated with brain can be recovered as dehydroascorbic acid.

TABLE III

The Effect of Different Amounts of Washed Heated Brain Preparation and Different Amounts of Ascorbic Acid

Incubated 120 minutes in air at 37°. The figures are in arbitrary units.

Brain preparation	Ascorbic acid	Colorimeter reading
ml.	*mg.*	
0.05	0.5	108
0.10	0.5	204
0.20	0.5	405
0.40	0.5	720
0.10	0.01	175
0.10	0.05	249
0.10	0.10	246
0.10	0.20	238

Since anaerobic incubation of brain and ascorbic acid yields no substance which condenses with PAB, it is reasonable to assume that dehydroascorbic acid is the oxidizing agent. It is apparently specific because methylene blue and quinone are inactive as measured by their effect on the oxygen uptake. The oxidation is not reversible since anaerobic following aerobic incubation does not reduce the color formation with PAB.

The copper ion added to the brain preparation and ascorbic acid inhibits the production of the compound which condenses with PAB. This fact brings up the possible relationship between copper and ascorbic acid. It would seem that metal ions present in the brain preparation do not normally play any part in the reaction because comparatively large amounts of cyanide do not affect the oxygen uptake and only slightly inhibit color production. It has been found, however, that occasionally copper added to the brain preparation without ascorbic acid produces the substance which gives the color with PAB and

this color has an absorption spectrum identical with that of the ascorbic cid product. This effect of copper is very sporadic, occurring not more than once in twenty different brain preparations and for no definable reason. Whe it
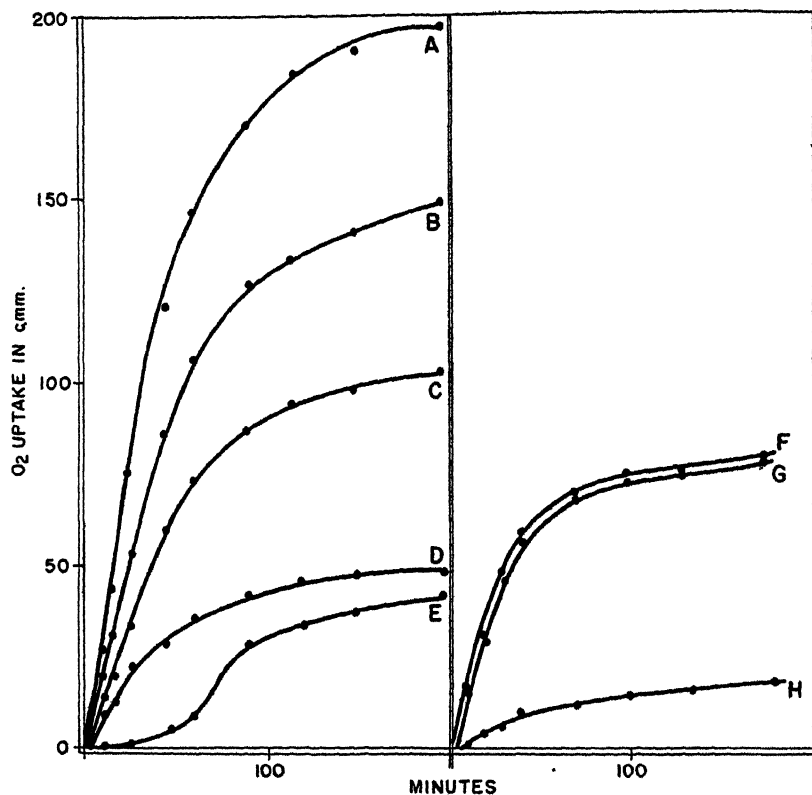
FIG. 1. The oxygen uptake of washed heated brain preparation in the presence of ascorbic acid at pH 6.7, 37°C. The brain preparation alone takes up no oxygen. Curves A, B, C, D: 0.8, 0.6, 0.4, and 0.2 ml. of brain preparation with 0.4 mg. ascorbic acid. Curve E: the autoxidation of 0.4 mg. ascorbic acid. Curve F: another brain preparation with either 0.2 or 0.4 mg. ascorbic acid. Curve G: brain preparation, 0.4 mg. ascorbic acid and 0.1 mg. NaCN. Curve H: brain preparation, 0.4 mg. ascorbic acid and 0.5 mg. epinephrine.

does occur, the copper increases the ascorbic acid effect, whereas, normally, as stated above, copper inhibits it. There is at present no explanation for this phenomenon.

Attempts were made to determine whether a substance which condenses with PAB occurs in plants. Peas were sprouted between layers of paper in peat moss. The roots and sprouts were ground and extracted with distilled water.

Addition of trichloroacetic acid and PAB to this extract gave a yellow color with the peak of the absorption spectrum shifted to the left of the one obtained with animal tissues. Incubation of the extract before the addition of PAB increased the color only slightly. The colorimeter values obtained with 0.5 gm. of pea roots at pH 7.0 were as follows: No PAB, 24; PAB on unincubated extract 175; after incubation 261. Incubation with catechol had a negligible effect.

DISCUSSION

Under normal conditions molecules of small molecular weight may be combined with tissue proteins. An example of this is histamine, which is apparently combined with a number of proteins through a peptide linkage (3). The experiments reported here indicate that brain protein (as well as that of several other organs but not that of blood) is combined with a compound which probably contains hydroxy groups attached to a ring. The linkage is stable because extensive washing and heating fail to break it. It is, however, very susceptible to oxidation in the presence of ascorbic acid which acts as a catalyst. The oxidation is accompanied by the splitting of the compound from the protein and the formation or exposure of an aldehyde group. The concentration of the compound in the same organ of animals of the same species varies a great deal (cf. frog muscle in Table II) which indicates a rapid metabolism. *In vitro* the reaction is rapid; a detectable amount of the compound is produced within a few minutes of the addition of the ascorbic acid to the brain preparation. The possible pharmacological properties of the compound, its chemical nature, and its status in the scorbutic animal are questions which await further investigation.

SUMMARY

1. A number of tissues, in particular, brain, liver, and kidney, incubated aerobically *in vitro* as slices or ground suspensions produce a compound which combines with *p*-aminobenzoic acid in acid solution to form a yellow color.

2. A study of this reaction in rat brain has shown that this compound can be produced when washed boiled brain protein is incubated aerobically with ascorbic acid. The latter acts as a catalyst to break the linkage between the protein and the compound. Oxygen is taken up in the process.

3. A number of aromatic hydroxy compounds such as epinephrine and catechol inhibit the reaction. Cyanide has little or no effect. No reaction occurs anaerobically.

4. The occurrence of the reaction in some animals has been described.

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THE BEHAVIOR OF THE NUCLEIC ACIDS DURING THE EARLY DEVELOPMENT OF THE SEA URCHIN EGG (ARBACIA)*

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It was found in earlier investigations by Masing (1, 2), by Brachet (3), and by Schmidt (4) that the eggs of *Arbacia* and other sea urchins contain large amounts of nucleic acids. During the early stages of development (up to the gastrula stage) the increase of the total nucleic acid content of the embryo is small in comparison to the initial nucleic acid content despite the formation of a large number of cell nuclei. In these respects the sea urchin eggs differ fundamentally from avian eggs which contain only extremely small amounts of nucleic acids before fertilization but form large amounts of these compounds during their development.

Brachet observed that unfertilized sea urchin eggs give only very weak Dische tests (5) but very strong furfural tests after being heated with strong hydrochloric acid. He concluded from these facts that the nucleic acid of the unfertilized sea urchin eggs (*Paracentrotus*) is mainly ribonucleic acid. This interpretation is confirmed in the present paper by the application of improved analytical methods. (An earlier statement to the contrary by Schmidt (4) who inferred the presence of desoxyribonucleic acid from a positive Feulgen test of the nucleoprotein fraction of unfertilized *Arbacia* eggs was erroneous.)

On the basis of ribose and desoxyribose determinations at various stages of the early embryonic development, Brachet suggested the assumption that ribonucleic acid was gradually transformed into desoxyribonucleic acid during the early development of the sea urchin eggs (3, 7).

Since the analytical methods available for the determination of nucleic acids have been greatly improved in the meantime, we undertook a reinvestigation of this problem with more recent analytical procedures. The results of this investigation are reported in the present paper.

EXPERIMENTAL

Suspensions of *Arbacia* eggs were divided into several batches each containing an equal number of eggs per ml. In addition, an aliquot of the original suspension was set aside for the determination of the number of eggs per ml. The counts were carried out in a cell count chamber with the undiluted egg suspensions. All batches except one were then fertilized under comparable conditions. At the desired stage of development each batch was homogenized in a Waring blendor after the addition of an equal volume of 10 per cent trichloroacetic acid. By means of this treatment it is possible to achieve the complete mechanical disintegration of the eggs which is an important prerequisite for the quantitative extraction of the non-nucleic acid P fractions.

The analytical procedure used for the determination of the nucleic acids and phosphoproteins was essentially that of Schmidt and Thannhauser (6). One important modification, however, was introduced into the original technique: Instead of calculating the amount of desoxyribonucleic acid P as the difference between the total P and the ribonucleic acid P, we determined the desoxyribonucleic acid P directly in the precipitate obtained on acidification of the alkaline hydrolysate. The precipitate was washed three times with a mixture of 0.5 N hydrochloric acid and 5 per cent trichloroacetic acid and then ashed for the determination of the total P.

The precipitation of desoxyribonucleic acid and the handling of the precipitate was facilitated by adding 1 ml. of a 1 per cent solution of egg albumen to each 5 ml. of the alkaline digest. It is advisable to do this whenever the DNA-containing fraction fails to precipitate in flocculent form.

The modification just described has the advantage of replacing the differential determination of the desoxyribonucleic acid P by its direct determination. Since the amounts of desoxyribonucleic acid in *Arbacia* eggs as well as in many other animal cells are much smaller than those of the ribonucleic acid the accuracy of the direct determination is considerably higher than that of the differential determination.

It was demonstrated in control experiments with sperm that the amounts of the desoxyribonucleic acid and of the ribonucleic acid introduced with the sperm were small in comparison to those present in the unfertilized eggs. In one experiment the amount of the sperm ribonucleic acid was 0.9 per cent of that of the eggs, the amount of desoxyribonucleic acid 3 per cent of that of the eggs.

RESULTS

Table I demonstrates the result of a representative experiment. The data obtained in seven other experiments on different batches of *Arbacia* eggs are in essential agreement with those shown in Table I.

Table II shows the amounts of the nucleic acid and other P fractions in the unfertilized *Arbacia* eggs.

Purine N: Total P Ratio in the Ribonucleic Acid Fraction of Arbacia Eggs.—Unfertilized *Arbacia* eggs were homogenized in a Waring blendor and freed from acid-soluble and lipid P compounds as described (6). 400 mg. of the dry powder were refluxed with 5 ml. of a 2 per cent solution of sulfuric acid for 2 hours. The hydroly-

sate was made up to 10 cc. and filtered. From 5 cc. of the clear filtrate the purines were precipitated by the addition of a hot saturated solution of silver sulfate in 2 per cent sulfuric acid. After standing for 24 hours in the refrigerator, the silver precipitate was centrifuged, washed 3 times with a saturated solution of silver sulfate,

TABLE I

Amounts of Desoxyribonucleic Acid and Ribonucleic Acid in Arbacia in Early Embryonic Stages

Date of experiment	Embryonic stage	$\gamma \times 10^{-3}$ P per embryo	
		DNA	RNA
July 17, 1947	Unfertilized	0.6	22.0
	Morula (8 hrs.)	3.4	22.8
	Pluteus (24 hrs.)	10.7	21.5

TABLE II

P Fractions of Unfertilized Arbacia Eggs
$\gamma \times 10^{-8}$ P per egg

DNA	RNA	Phospholipid	Phosphoprotein	Total
0.9	21.3	35	2.5	96

and decomposed with 5 cc. N hydrochloric acid in a boiling water bath. In the supernatant from the silver chloride, the total N was determined according to Kjeldahl.

Total nucleic acid P: 0.44 per cent

Total purine N: 0.46 per cent

$$\text{Found:} \quad \frac{\text{Purine N}}{\text{Nucleic acid P}} : 1.05$$

$$\text{Calculated:} \quad \frac{\text{Purine N}}{\text{P}} \text{ in yeast nucleic acid: } 1.13$$

DISCUSSION

It was found that during the first 24 hours of the development of the *Arbacia* egg (pluteus stage), the amount of ribonucleic acid remains practically unchanged, whereas the amount of desoxyribonucleic acid steadily increases.

It follows from our analyses that the early development of the *Arbacia* egg involves a striking change of the mutual proportion of the two components of the nucleic acid fraction in favor of the desoxyribonucleic acid. The average ratio $\frac{\text{DNA}}{\text{RNA}}$ is 0.05 in the unfertilized egg, 0.17 in the morula (8 hours), and 0.46 in the pluteus (24 hours).

In this respect our observations are in essential agreement with those of Brachet. In our experiments, however, the change of the proportions between both nucleic acids is entirely caused by the increase of desoxyribonucleic acid per embryo, while Brachet found not only an increase of the amount of desoxy-pentose, but also a very considerable decrease of the total pentose per gram of dried embryos. Brachet interpreted this decrease of the total pentose per gram of embryos as being caused by the decrease of ribonucleic acid. This interpretation is not compatible with the results of our determinations of ribonucleic acid.

It should also be noted that Brachet relates his figures to the unit of weight while our values represent those per embryo. It appears that only the latter method of calculation permits an answer to the question as to whether the amount of a substance in a developing egg actually changes.

The observations presented in this paper demonstrate that the nucleic acids of the developing sea urchin egg cannot be considered as a closed metabolic system in the sense that the intense synthesis of desoxyribonucleic acid would proceed at the expense of the ribonucleic acid accumulated in the mature egg. They show that this synthesis is accompanied by a corresponding rise of the total nucleic acid P for which the new formation of desoxyribonucleic acid is exclusively responsible, whereas the amount of ribonucleic acid per embryo remains practically unchanged. It is obvious that the rise of the total nucleic acid fraction could not be detected in some of the earlier investigations (1, 3) since its extent during the early phases does not exceed the range of analytical errors due to the fact that the desoxyribonucleic acid represents only a very small fraction of the total nucleic acid P in the mature egg.

Thus, the formation of new nuclei during the cleavages of the sea urchin egg involves the formation of nucleic acid from other cell constituents. Since the total nucleic acid P in the mature *Arbacia* egg amounts merely to approximately 25 per cent of the total P there is an abundance of possible P sources available for the synthesis of nucleic acids. Whether or not the metabolic pathway of the biological synthesis of desoxyribonucleic acid goes through an intermediary stage of ribose compounds is still an open question the answer to which is beyond the scope of the present investigation.

From the biological point of view it should be emphasized that the presence of a large excess of RNA in the nucleic acid fraction of the unfertilized sea urchin egg can no longer be considered as an exceptional occurrence. It has recently been shown by Davidson and Waymouth (8), by Schmidt and Thannhauser (6), and by Schneider (9) that ribonucleic acid is quantitatively predominant in the nucleic acid fraction of many adult animal tissues.

SUMMARY

1. The unfertilized *Arbacia* egg contains an average of $20\gamma \times 10^{-8}$ ribonucleic acid and 0.7 to $1\gamma \times 10^{-8}$ desoxyribonucleic acid.

2. During the first 24 hours of development, the amount of ribonucleic acid per embryo remains practically unchanged whereas that of desoxyribonucleic acid steadily increases. At the end of this period, the amount of desoxyribonucleic acid per embryo is 10 to 15 times larger than that of the unfertilized egg.

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METHOD FOR OBTAINING KNOWN LOW CONCENTRATIONS OF MUSTARD (H) OR LEWISITE (L) GAS IN AIR*‡

BY JOHN H. NORTHROP

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High concentrations of H, L, or other gases in air may readily be obtained by regulating the flow of air through the liquid gas, itself, or through a solution of the gas in some inert solvent. Very low concentrations, in the range of a few micrograms of gas per liter of air, are difficult to obtain by these methods. The "diffusion tube" described in this paper offers a convenient and accurate method for obtaining such low concentrations of gas.

The diffusion of a gas through a porous plug is independent, within wide limits, of the flow of air past the plug. Such "diffusion tubes" may be readily prepared of any desired dimensions by fusing disks or rods cut from alundum[1] into the end of pyrex tubes. An alundum disk 5 mm. in diameter and 3 mm. thick allows 2 to 3 micrograms of H or 10 to 20 micrograms of L to diffuse in 1 hour at 20°C.

Preparation of the Tubes.—A piece of pyrex tubing of the desired diameter and about 10 cm. long is fused together at one end. A disk is cut from the alundum plate which will fit tightly into the open end of the tube. A little powdered alundum, to absorb the liquid and prevent it from flowing up the sides of the tube, is placed in the bottom of the tube. A few drops of liquid L or H are placed on the powdered alundum in the bottom of the tube, care being taken to avoid contamination of the sides. The alundum plug is inserted in the open end of the tube. The end of the tube is then heated with an oxygen blast lamp until the glass fuses to the alundum disk. A fine wire with a loop is attached to the upper end of the tube so that it may be suspended in a flask (Fig. 1).

Standardization.—The tubes are suspended in an air stream of about the same rate of flow as that to be used. The air is bubbled through dilute sulfuric acid

until sufficient gas is collected to give an accurate titration. The gas is then titrated as described in another article.[2]

Sources of Error

Temperature.—The vapor pressure of H and L changes rapidly with temperature so that for accurate results the temperature must be kept constant.
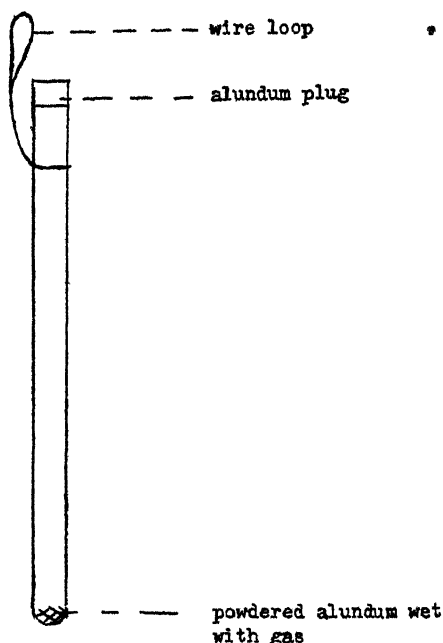
FIG. 1. Diffusion tube for the liberation of small constant quantities of H or L. One-half actual size.

Pressure.—Sudden fluctuations in pressure result in forcing air in and out of the diffusion disk. Such fluctuations in pressure may arise if the air is bubbled through liquids under pressure. This difficulty may be avoided by drawing the air through the flask containing the diffusion tube. Under these conditions the pressure in the flask containing the diffusion tube remains constant.

Flow of Air Past Diffusion Tubes.—Violent air currents impinging directly on the diffusion disk might cause some convection currents in the plug and so increase the yield. For strictly accurate results larger and thicker disks could be used. With air flow in the range used in the present work, however, no effect

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[2] Northrop, J. H., Titration of small amounts of mustard and other gases with bromine and methyl red, OSRD Formal Progress Report, No. 516, April, 1942.

of the flow of air can be detected.   The results of experiments to test this point are shown in Table I.   It is necessary to use *dry* air with L tubes.

Condensation of Gas on Outside of Tubes.—If the tubes are kept in a closed container when not in use, gas will condense on the outside of the glass and the

TABLE I

Standardization of Diffusion Tubes with Varying Rates of Air Flow

H Tube No. 6.   Disk 5 mm. diameter, 3 mm. thick made of alundum, R A 84, Norton Alundum Co.

Flow of air past disk	Time	Total H delivered	H per hr.
cm. per sec.	*hrs.*	*micrograms*	*micrograms*
0	1.0	2.1	2.1
0	5.0	10.0	2.0
0	24.0	50.0	2.1
7.5	1.0	1.8	1.8
		2.3	2.3
	10.0	25.0	2.5
	10.0	15.0	1.5
0.12	2.0	5.0	2.5
		3.1	1.5
		4.0	2.0

L Tube No. 5.   2 mm. × 15 mm.

		Total L delivered	L per hour
		micrograms	*micrograms*
0	24.0	20.0	0.8
0.10	0.5	0.5	1.0
7.5	0.5	0.4	0.8

concentration of gas will be much too high, when the tubes are first used.   If the tubes are to be used frequently, they should be kept in a slow stream of *dry* air.   Under these conditions the correct quantity of gas is obtained at once.

SUMMARY

Constant small quantities (0.1 to 100 micrograms per hour) of mustard (H) or lewisite (L) gas may be obtained by allowing the vapor to diffuse through a porous alundum plug of suitable dimensions.   By regulating the rate of flow of air past the plug known concentrations of the gases in air may be obtained.

A CONVENIENT METHOD FOR POTENTIOMETRIC TITRATION OF CHLORIDE IONS*

By JOHN H. NORTHROP

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The potentiometric titration of chloride ions as usually carried out is accurate but rather time-consuming and is not suitable for the determination of very small quantities.

The method described in the present paper can be used to determine small amounts of 10^{-4} molar, or stronger, chloride solutions with an accuracy of about $\pm$ 0.03 ml. 10^{-4} molar silver nitrate. The titration requires only a few minutes and a series of solutions may be titrated consecutively.

Solutions and Apparatus Required.—All reagents should be chloride-free if possible.

0.25 M potassium nitrate.
10^{-3} M silver nitrate made up in 0.25 M potassium nitrate.
Calcium carbonate.
0.10 M sodium oxalate, 0.001 M silver nitrate, 0.1 M potassium nitrate (14 gm. sodium oxalate, 10 gm. potassium nitrate, and 10 ml. of 0.1 M silver nitrate in 1 liter).
2 silver electrodes.
Galvanometer: 0.5×10^{-6} amperes per mm. or more sensitive.
2 ml. microburette graduated in 0.01 ml. (E. Machlett & Son, New York, No. A8-525).

Arrangement of Apparatus.—*Preparation of Reference Electrode:* A hole about 5 mm. in diameter is blown in the bottom of a 1×10 cm. pyrex test tube and the tube packed with glass wool and hyflo filter-cel (Johns-Manville Corporation) as shown in Fig. 1. The tube is filled with 0.1 M sodium oxalate,[1] 0.001 M silver nitrate, 0.1 M potassium nitrate and some of the solution drawn through the filter-cel by suction. The tube is then filled completely with the solution

[1] The use of silver oxalate as a reference electrode was suggested by Dr. Edgar L. Eckfeldt of the Leeds and Northrup Company.

and the stopper and silver electrode forced in place. The stopper should be sealed or taped so as to prevent any air from leaking in.

Silver Electrodes: Silver foil about $1 \times 5 \times 0.1$ cm. welded to No. 12 silver wire. Polish with fine emery paper and short circuit in 0.25 M potassium nitrate, 10^{-4} M silver nitrate for 24 hours. The reference electrode should remain in good condition indefinitely but the titrating electrode may lose sen-
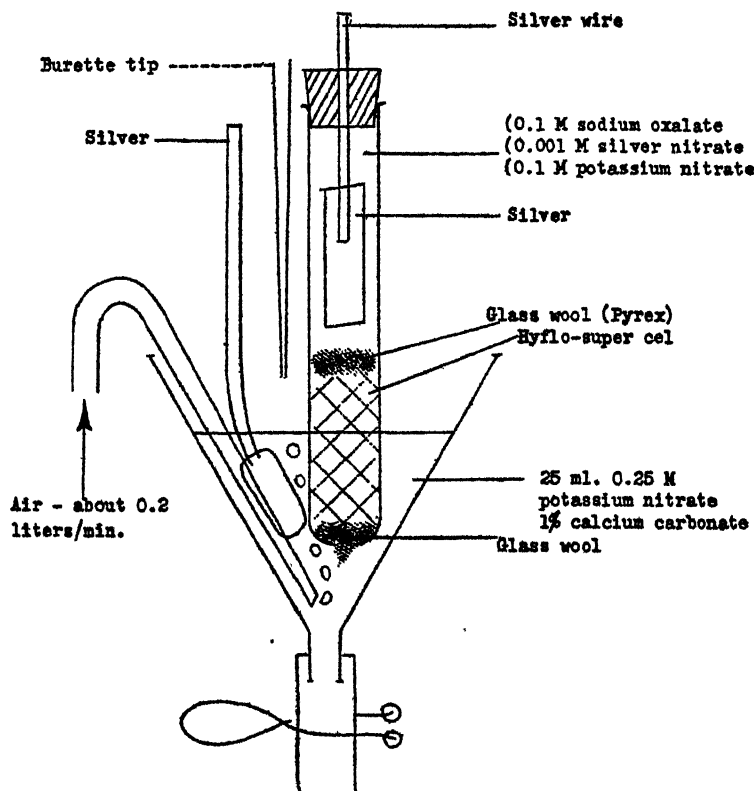
FIG. 1

sitivity so that equilibrium is reached too slowly. The sensitivity can be restored by polishing with emery cloth. This treatment causes the electrode to be negative for a few minutes and the first few titrations may be incorrect.

The titrating electrode should be bent in a cylinder and placed as low as possible in the funnel. It may be held in place by a rubber band on the reference cell.

Operating Directions.—Set up apparatus as in Fig. 1. Cover the silver electrode with 0.25 M potassium nitrate, calcium carbonate (about 10 ml.). Connect electrodes to galvanometer and put 2,000 to 3,000 ohms in parallel with galvanometer. The titrating electrode should be negative. Add about 1 ml.

10^{-3} M silver nitrate. The titrating electrode should now be strongly positive. Add 10^{-3} molar chloride until the galvanometer reaches 0. There is usually a slight drift for a few minutes after which the galvanometer should remain constant. Chloride or silver ions are then added until the galvanometer remains constant at 0.

Titration.—Add 1 to 10 ml. of solution containing chloride ions to cell. Titrating electrode becomes negative. Run in silver nitrate until galvanometer returns to 0 and remains constant at 0 for at least 30 seconds. With sensitive electrodes the titration should require about 1 minute with 10^{-3} M or more concentrated silver nitrate, and 2 to 3 minutes with 10^{-4} M silver nitrate.

<div align="center">TABLE I</div>

	0		10^{-4}			10^{-3}	
Cl taken—concentration, *mols/liter*							
Volume of sample, *ml.*	0.5	1.0	0.25	0.5	1.0	0.5	1.0
Total volume in cell, *ml.*	8–10						
Silver nitrate concentration, *mols/liter*	10^{-4}		10^{-4}			10^{-3}	
Blank	0.08	0.16					
Silver nitrate (corrected for blank), *ml.*			0.25	0.48	1.04	0.49	1.02
			0.29	0.40	1.10	0.50	1.00
			0.22	0.52	1.04	0.48	0.99
			0.24	0.52	1.00	0.50	1.02
Average			0.25	0.48	1.04	0.49	1.01

Subsequent Titration.—Do not empty cell but merely drain off solution until titrating electrode is just covered. This should not change galvanometer reading. If it does the galvanometer should be adjusted to 0 again before the next titration.

Blank for Change in Volume.—When titrating with 10^{-4} M silver a small titration is required when 1 ml. water is added. This blank titration is subtracted from the titration of the sample.

If 10^{-3} M or stronger silver nitrate is used a larger volume of solution may be kept in the cell. No blank titration is found with 10^{-3} M or stronger silver nitrate.

The results of some titrations of hydrochloric acid and various concentrations of silver nitrate are shown in Table I.

<div align="center">SUMMARY</div>

A convenient reference cell and method of potentiometric determination of chloride ions with silver nitrate is described. The method is accurate to about ± 0.02 ml. 10^{-4} M silver nitrate.

SOME CORRELATIONS BETWEEN DEVELOPMENT AND RESPIRATION IN THE SAND DOLLAR EGG, AS SHOWN BY CYANIDE INHIBITION STUDIES*

By W. A. ROBBIE

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Invertebrates show considerable variation in the enzymatic mechanisms involved in energy release: certain species depend partially or entirely upon anaerobic processes, while other forms require molecular oxygen for survival. The eggs of the sand dollar, an echinoderm, are especially sensitive both to oxygen lack and to inhibition of respiration and cell division by cyanide. Their large size and the rapid morphological and metabolic changes that occur during cleavage make them favorable material for a study of the interrelationship between cellular respiration and development.

Where enzymatic oxidations are catalyzed by heavy metal systems, the use of cyanide inhibition as an analytical tool in studying intracellular processes has certain definite advantages: the agent is active in low concentrations, it has a relatively specific action, it penetrates cell membranes readily, and with proper precautions the concentration may be controlled quantitatively (1). Although cyanide is perhaps not always equivalent to low oxygen tension in its effects, it is easier to use with precision, and the experimental approach is therefore broadened. The following paper presents results of certain experiments with cyanide on sand dollar eggs and sperm, and a discussion of the relationship of cell respiration to certain other aspects of developmental metabolism.

Material and Methods

Eggs of the sand dollar, *Echinarachnius parma*, were obtained and studied at the Mount Desert Island Biological Laboratory at Salisbury Cove, Maine, during the month of August, 1946. The oral portion of the shell of the adult animal was removed by cutting entirely around the edge (2), and the oviducts and ovaries were carefully removed with curved forceps and placed in a bowl of filtered sea water. After a few minutes the mature eggs had streamed out and the remaining material was strained off by pouring the suspension through a fine cloth. Egg volumes for the manometric determinations of oxygen consumption were measured by centrifuging the unfertilized egg suspension in a hematocrit tube for 5 minutes in a small clinical centrifuge. Because of unavoidable variations in speed and in the volume of the egg jelly the actual amounts of the egg material varied somewhat from one experiment to another, but the

results are always expressed in terms of a control from the same lot as the experimental samples. In most experiments 90 per cent or more of the eggs were fertilizable.

Sperm was left within the shell until it was needed; a dilute sea water suspension was prepared just before insemination.

The Warburg manometers of about 17 cc. volume, which were used for oxygen up-take measurements, were shaken at sixty cycles per minute in a constant temperature water bath at 20°C. Each flask received 3 cc. of 15 per cent egg suspension in sea water, a concentration which permitted maximum respiration. Six-tenths cc. of CO_2-absorbing solution was placed on two filter papers in the center well. The sea water was left unbuffered, and tests with indicator at the end of the experimental periods showed that there had not been an appreciable change in pH.

In the manometric experiments with cyanide, $Ca(CN)_2$-$Ca(OH)_2$ mixtures were used in the center wells (1). The effect of cyanide on development was determined

TABLE I

A Comparison of Three Methods of Exposing Echinarachnius Eggs to HCN Gas in Cell Division Studies

The three methods were as follows: (1) $Ca(CN)_2$-$Ca(OH)_2$ mixtures in the center wells of manometer flasks; (2) eggs in vials in HCN-sea water; (3) a drop of egg suspension in sea water exposed in a covered dish to 30 cc. of HCN-sea water solution.

HCN concentration	Stage of development after 5½ hrs. exposure		
	(1) Manometers	(2) Vials	(3) Dishes
0	Early blastula	Early blastula	64 cells to early blastula
1×10^{-6} M	8–16 cells	16–32 cells	16–64 cells
2.2×10^{-6} M	1–8 "	1–2 "	1–16 "
4.6×10^{-6} M	1 cell	1 cell	1 cell

by observing eggs which had been treated in three ways: (1) exposed in the manometer flasks, (2) exposed in HCN-sea water in small stoppered vials, and (3) exposed to HCN gas in a covered dish with a small droplet of egg suspension in contact with the gas from a large volume of HCN-sea water (3). A comparison of the results obtained by these three techniques is presented in Table I.[1]

EXPERIMENTAL RESULTS

(a) *Respiration*

Change in Rate at Fertilization.—The *Echinarachnius* egg resembles that of the sea urchin in showing an increase in the rate of respiration at the time of

[1] The uniformity in effect of a given concentration of cyanide on cell division, as shown in Table I, is a check of the correctness of the HCN equilibrium between the sea water and the $Ca(CN)_2$-$Ca(OH)_2$ center well solutions. Since certain of the listed concentrations are beyond the limits of the micro test for cyanide used in formulating the balanced center well solutions (4), a biological test of this sort is necessary.

fertilization. The course of oxygen consumption of unfertilized and fertilized eggs is indicated in Fig. 1, and is quite similar to that found for the Pacific Coast sand dollar, *Dendraster excentricus* (5). The oxygen uptake of the unfertilized eggs is usually from one-half to one-fourth that of the fertilized ones.

Cyanide Inhibition of Oxygen Consumption.—Egg respiration is depressed by cyanide, even at low concentrations. As shown in Fig. 2, there is a significant inhibition of the oxygen consumption of fertilized eggs with a cyanide
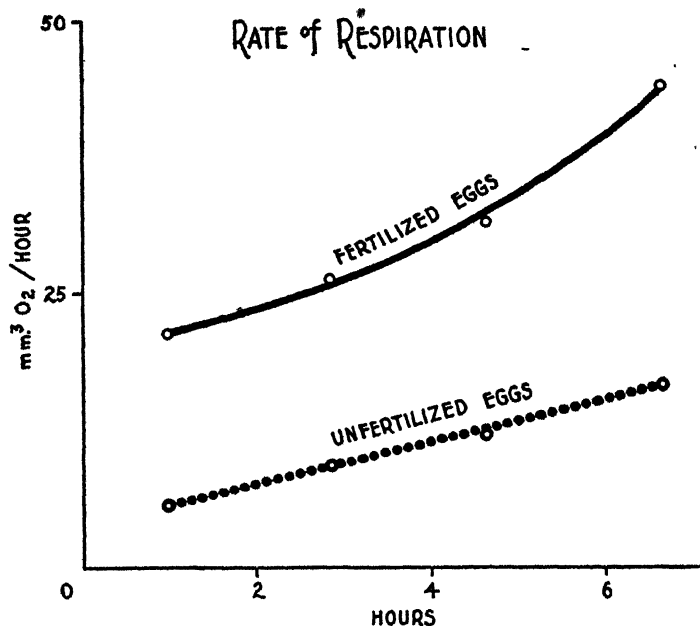
FIG. 1. Comparison of the respiratory rates of unfertilized and fertilized *Echinarachnius* eggs. Each manometer flask received 3 cc. of 15 per cent (by volume) egg suspension.

concentration as low as one-millionth molar. Inhibition of the respiration of unfertilized and fertilized eggs by a range of HCN concentrations from 10^{-2} to 10^{-7} M is shown in Fig. 3. On a percentage basis the unfertilized eggs are apparently somewhat less sensitive than the fertilized ones. Fifty per cent inhibition of the respiration of fertilized eggs occurs at a cyanide concentration of about 10^{-6} M HCN, indicating that these eggs are of the same order of sensitivity as those of *Asterias* (unpublished observations), and are somewhat more sensitive than those of the sea urchin (6).

Fig. 4 indicates the course of oxygen consumption when fertilized eggs are shaken in manometers in a marginal concentration of HCN, 10^{-6} M. The degree of inhibition is consistent for a 10 hour period.

Recovery of Respiration after Cyanide Exposure.—When fertilized eggs were exposed to 2.2×10^{-6} M HCN (a concentration sufficient to depress the respir-
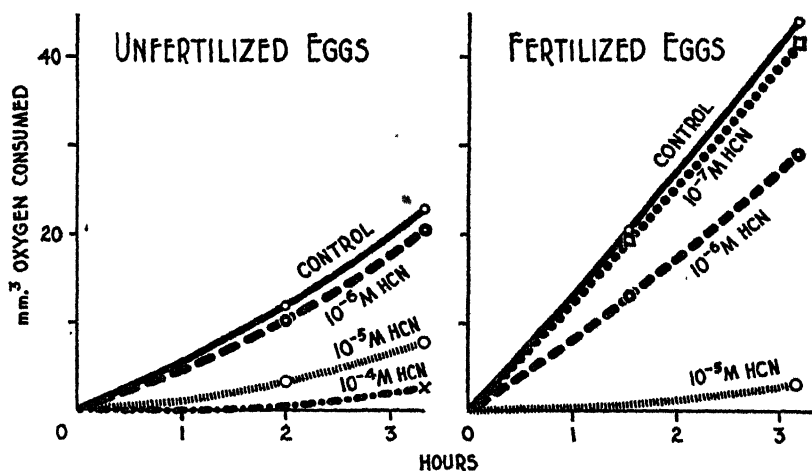
FIG. 2. Inhibition of the oxygen consumption of sand dollar eggs by HCN.   Ca (CN)₂-Ca(OH)₂ solutions were used in the center wells.
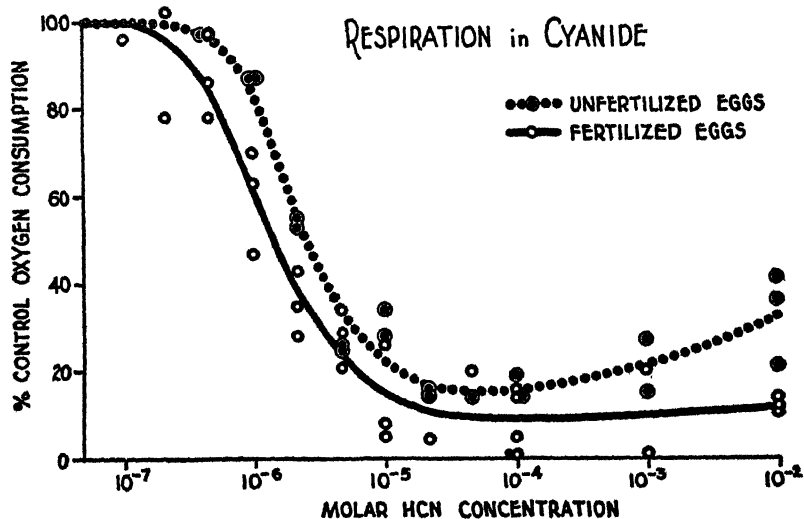
FIG. 3. Variation in the sensitivity of the respiration of sand dollar eggs with cyanide concentration.   Each point is for a 2 hour determination period.

ation 65 per cent), for periods of 5, 20, and 60 minutes, the recovery was rapid after removal of the cyanide.   By the time the eggs were washed and the

manometers were reequalibrated, the oxygen consumption had returned to the control level. Both the unfertilized and the fertilized eggs showed only partial recovery after 10 minute exposures to 10^{-4} M HCN. A similar treatment with 10^{-2} M cyanide was irreversible and resulted in disintegration of the eggs.

Effect of Cyanide on Sperm Respiration.—Inhibition of the oxygen consumption of sand dollar sperm by HCN is demonstrated in the curves in Fig. 5. A concentration of only 10^{-6} M HCN is sufficient to cause 65 per cent depression. The respiration of mammalian sperm has been shown to be inhibited
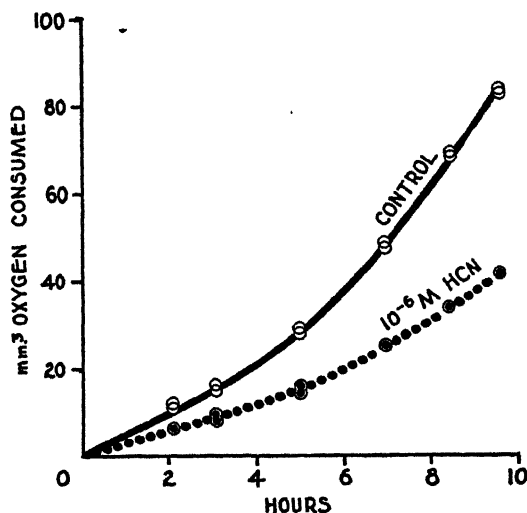
FIG. 4. Respiration of fertilized sand dollar eggs in a marginally effective solution of HCN (10^{-6} M). Each of the four manometer flasks contained 3 cc. of 5 per cent egg suspension.

by cyanide (7), but little quantitative work on invertebrate sperm has been reported.

(b) Fertilization and Cell Division

Cyanide Treatment of Unfertilized Eggs.—Table II indicates the effect of exposure to cyanide before fertilization. Eggs were exposed to various concentrations of HCN for 7 minutes, washed in sea water for 8 minutes, and then untreated sperm was added. Normal fertilization membranes appeared, even on those eggs that had been exposed to high concentrations of cyanide. Cleavage rate was unaffected by the treatment with 10^{-3} M HCN, although only one-tenth this concentration was sufficient to prevent all development when the sperm rather than the eggs was similarly exposed (Fig. 6).

Exposure of Sperm to HCN.—The effect of cyanide on the motility of sand dollar sperm is illustrated in Fig. 6. By correlation with Fig. 5 it appears
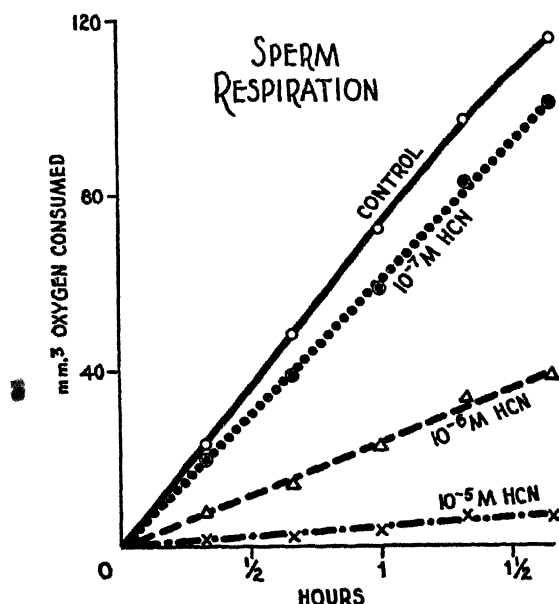
FIG. 5. Depression of the oxygen consumption of a dilute sperm suspension by various concentrations of HCN.

TABLE II

Effect of 7 Minutes' Exposure of Unfertilized Echinarachnius Eggs to HCN on Subsequent Fertilization and Development

Observations on development were made 3½ hours after the eggs were removed from the cyanide solutions.

HCN concentration	Fertilization	Cytolysis	Development 3½ hrs. later
	per cent	*per cent*	
0.01 M	95	35	1–2 cells
0.0046 M	95	25	1–4 "
0.0022 M	95	0	4–8 "
0.0010 M	95	0	8–16 "
0	95	0	8–16 "

that 30 per cent residual respiration is sufficient to maintain normal activity. Fig. 6 indicates also the relationship of motility to fertilizing capacity. Sperm was left in various HCN-sea water solutions for 5 minutes, and then small drops of each of the sperm suspensions were added to untreated eggs in large volumes of fresh sea water. After 4 hours the eggs were examined and the

extent of development noted.   In general there was a close correlation between the kinetic activity and the developmental potentiality of the sperm, and at a concentration of 10^{-4} M HCN the treatment became definitely irreversible. Although exposure of the sperm to a concentration of HCN just sufficient to arrest motility permitted normal fertilization, this may have resulted from dilution of the cyanide and recovery of activity when the sperm was added to the fresh sea water containing the eggs.   Mammalian sperm exposed to 10^{-3} M HCN showed complete reversibility (7).
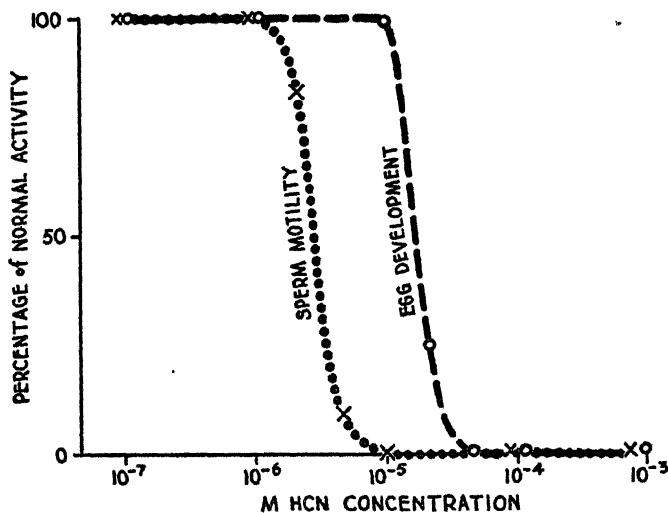
FIG. 6. Effect of exposure of sperm to cyanide on sperm motility and egg development.   For details see text.

Effect of Cyanide on Cleavage.—Cleavage provides an easily observable index of developmental metabolism that is useful in correlating respiratory and morphogenetic processes.   Dependence of cell division upon respiration varies greatly with different species; *Echinarachnius* is particularly sensitive to both oxygen lack and HCN interference with enzyme function.   Fig. 7 shows the critical range of cyanide concentrations which is effective in retarding or suppressing cleavage. 10^{-6} M HCN depresses the rate about 50 per cent, and at ten times this concentration the oxygen consumption is reduced to a minimal level and development is completely arrested.   Cell division in the sand dollar is thus somewhat more sensitive to inhibition by HCN than is the process in *Arbacia* (6).

At a marginally effective concentration of 10^{-6} M HCN there is extreme individual variation in the response of the exposed eggs.   The oxygen consumption of control and treated samples is shown in Fig. 4.   After 5 hours of shaking in the manometer flasks the control eggs had developed almost uniformly to

the 128 cell stage, but those in cyanide were quite irregularly retarded.   Some of the eggs had undergone no division while others were almost as far along as the controls.   At 10 hours the situation was similar: the controls were nearly all at the blastula stage and those in cyanide varied from one cell to early blastula.   The situation is somewhat similar to that observed by Tyler (8) at temperatures low enough to interfere with cytoplasmic division but which still permitted nuclear division to proceed.   He found considerable variation in the response of individual eggs to the low temperature: although some showed complete inhibition, others divided at an easily measurable rate.
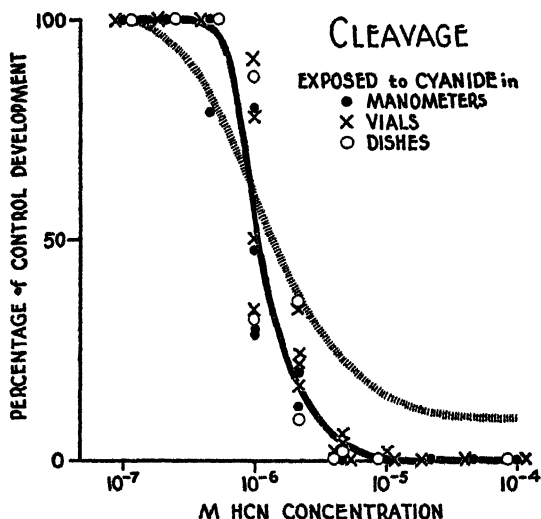
FIG. 7. Inhibition of cleavage in the sand dollar egg by cyanide.   Exposures were made as indicated by the symbols with the three techniques described in Table I. The shaded curve represents the oxygen consumption as shown in Fig. 3.

The division of the cell is quickly interrupted by 10^{-2} M cyanide (Fig. 11 d). Eggs that were placed in this concentration a few minutes before the start of the actual division were most of them still undivided 5 hours later.   A few had attempted to separate and remained fixed in a dumbbell shape with incomplete separation of the two blastomeres.   A similar treatment with 10^{-5} M HCN resulted in incomplete arrest of cell division, but the eggs were capable of dividing only a few times in a very irregular fashion.   An example of the incomplete division which was frequently seen is shown in Fig. 11 c.

(c) Cytolysis

Echinarachnius eggs display a peculiar breakdown of cellular organization under certain conditions of cyanide exposure, and the apparent relationship

of this behavior to the rate of oxygen uptake and the stage of development suggests that a metabolic factor of some significance may be involved.

Appearance of Cytolyzed Eggs.—Two hours or so after fertilized eggs are placed in a 10^{-4} M HCN solution the plasma membrane becomes covered with tiny irregularities which slowly increase in size until the membrane is covered with transparent blebs. This is shown in Fig. 11 *a* and *b*. A similar "blister" or "pseudopodia" formation was observed by Runnström (9) on sea urchin eggs that were left in N/5000 KCN for 4 hours.
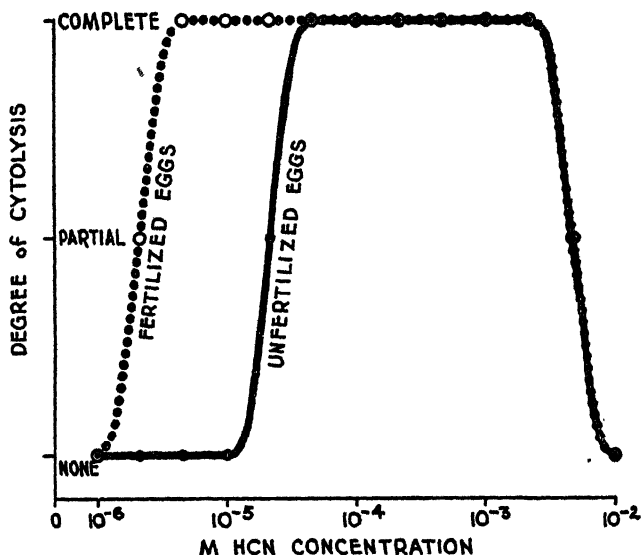
FIG. 8. Extent of cytolysis in unfertilized and fertilized sand dollar eggs after 5 hour exposures in the cyanide solutions represented on the abscissa.

The process seems to involve a separation of the membrane and an extrusion of clear fluid into the outpocketings that are formed. The change is not confined to the surface since the interior of the egg is also broken up into round globules. The size of the droplets and the extent of the cytolysis vary with conditions, but no cell which shows droplet formation appears to proceed further in morphological development.

Variation with Cyanide Concentration.—Fig. 8 indicates the relationship between the incidence of cytolysis and the concentration of the cyanide solution. 10^{-2} M HCN stops all morphological change quickly, and preserves the eggs in such a way that they retain their original appearance for hours. But reducing the concentration only slightly brings about cytolysis of some of the eggs, and in the range including 2.2 × 10^{-3} M to 4.6 × 10^{-5} M all of the eggs

are completely broken down to the droplet form. Below this range fertilized eggs are considerably more sensitive than those that have not been fertilized: the marginal effective concentration of HCN for the fertilized eggs is only one-tenth of that for the unfertilized ones.

Change in Sensitivity with Development.—There is a marked increase in sensitivity to cytolysis by cyanide as the egg develops. The most resistant eggs were the immature germinal vesicle forms that were occasionally found. Irrespective of the concentration or length of exposure they did not show droplet formation.
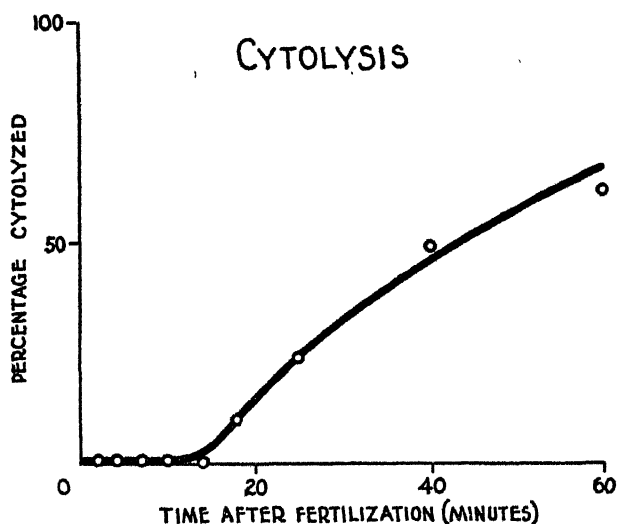
FIG. 9. Cytolysis of fertilized eggs on exposure to 10^{-5} M HCN, showing increasing sensitivity as development proceeds. Eggs were placed in cyanide at the time after fertilization indicated on the abscissa, and were examined 5 hours later to determine the extent of breakdown.

Unfertilized eggs may remain in 10^{-5} M HCN solution for a long time with no alteration in either their appearance or their capacity for development. After fertilization the sensitivity increases gradually. As shown in Fig. 9, eggs placed in 10^{-5} M cyanide at 14 minutes after fertilization were not cytolyzed 5 hours later, but there was a progressively increasing amount of breakdown if a longer period of normal development was allowed before exposure to cyanide was started.

Fig. 10 demonstrates again the increase in sensitivity to cytolysis that accompanies development. Eggs at the 64 cell stage at the time of treatment were completely cytolyzed when they were examined 20 hours after a 30 minute exposure to 10^{-4} M HCN. A similar 30 minute exposure of 1 cell eggs

permitted development only to the 2 cell stage or slightly beyond but did not produce any cell breakdown.

Resumption of development by fertilized eggs after removal from low concentrations of HCN may lead to cytolysis, whereas continuous exposure preserves the eggs intact. For example, in an experiment in which eggs were placed in 10^{-5} M HCN 5 minutes after fertilization and washed 1 hour later, half were cytolyzed 4 hours later and all were broken down at 7 hours. Those which were in the cyanide continuously remained unchanged. Only one-third of the eggs which were removed from the cyanide had been able to
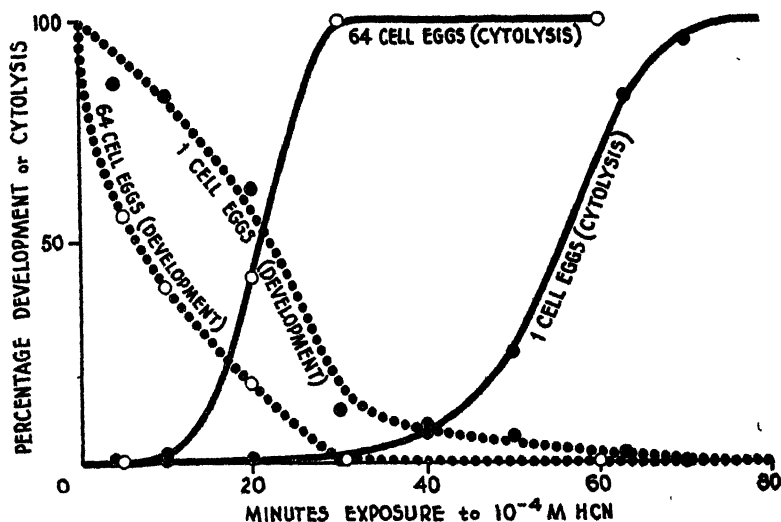
FIG. 10. Effect of various exposures to 10^{-4} M HCN on subsequent development and cytolysis of sand dollar eggs. 1 cell eggs were exposed to cyanide at 12 minutes after fertilization; 64 cell eggs were in cyanide at 5 hours after fertilization. Eggs were examined 20 hours after removal from cyanide.

complete the first cleavage division at the time that the controls had progressed to the 32 cell stage. In contrast to this, unfertilized eggs exposed to this concentration retained normal developmental capacities.

Cytolysis by Heat.—Öhman (10) observed droplet formation on the outer surface of sea urchin eggs that were exposed to 35 to 45°C. temperatures. An experiment with sand dollar eggs showed that heat was a relatively ineffective way of producing the cytolysis in this form. Both unfertilized eggs and eggs 15 to 20 minutes after fertilization were heated for 5 minute periods at various temperatures from 30 to 50°C. and the subsequent appearance noted. After treatment at the lower temperature the fertilized eggs developed normally, but raising the exposure temperature to 34°C. resulted in complete

cessation of development (sea urchin eggs respond similarly (10, 11)). After treatment at 50°C. the fertilized eggs showed a few small droplets at the plasma membrane and although these gradually enlarged they did not become as extensive as those produced by cyanide treatment. The unfertilized eggs showed no change as a result of the heat treatment.

Fig. 11 shows photomicrographs of eggs cytolyzed by cyanide.

(d) Lethal Dosage

Inhibition of respiration and cell division in the sand dollar egg by cyanide is in itself no evidence of irreversible change, since, as has been shown above, these processes may be resumed again when the cyanide is removed. To determine the lethal dosage it is necessary to observe either the disintegration of the eggs in cyanide or to note the lack of recovery after the HCN has been

TABLE III

Reversibility of a 1 Hour Exposure of Fertilized Eggs to Various Concentrations of HCN
Eggs were placed in cyanide one-half hour after fertilization.

HCN concentration	Percentage of respiration inhibited while in HCN	Appearance 30 hrs. after exposure
	per cent	
0	0	Early gastrulae
10^{-6} M	43	Irregular motile forms and some early gastrulae
10^{-5} M	84	A few irregular motile masses of cells
10^{-4} M	90	A few irregular swimming blastulae
2.2×10^{-4} M	90	A few blastulae with motile cilia, none swimming
4.6×10^{-4} M	90	" "
10^{-3} to 10^{-2} M	90	No division, all cells cytolyzed

washed off. If the temperature is constant the lethal dosage depends upon the concentration of cyanide, the duration of the exposure, and the stage of development of the egg.

Concentration of Cyanide.—Table III shows the results of an experiment on fertilized eggs with cyanide concentrations of 10^{-2} to 10^{-6} M, and an exposure period of 1 hour. The second column lists the inhibition of oxygen consumption observed for these concentrations (from Fig. 3). It is evident that only the lowest concentration used permits normal growth after this treatment period, but a much higher level of HCN is necessary to prevent all development.

Duration of Exposure.—Table IV presents the results of an experiment in which fertilized eggs were left for various times in a marginally effective concentration of cyanide, 2.2×10^{-6} M, and the subsequent development observed. After exposures of 6 hours or less some of the eggs were able to resume development, but if the exposure was longer the treatment was irreversible.

This sensitivity contrasts sharply with the behavior of *Arbacia* eggs, which develop after 24 hours in cyanide of almost one-thousand times this concentration (12).

Stage of Development.—The stage of development of the egg is an important factor in the determination of the reversibility of the cyanide effect. The unfertilized egg can tolerate for several hours a concentration of HCN that completely disrupts the organization of the fertilized egg. The fertilized egg in turn is much less affected during the early stages of the first cleavage division than at a more advanced stage (Fig. 9). In Fig. 10 the development of eggs treated with 10^{-4} M HCN at the 1 and 64 cell stages is compared. The advanced group is considerably more sensitive than the 1 cell eggs.

TABLE IV

Reversibility of Various Exposures of Fertilized Sand Dollar Eggs to a Marginal Concentration of HCN (2.2 × 10⁻⁶)

The eggs were placed in cyanide 1½ hours after fertilization.

Duration of exposure	Development after 30 hrs.
hrs.	
0	Swimming prism larvae
½–4	About ¼ swimming prism larvae, the rest retarded
6	A few swimming gastrulae
9	None developing, all eggs cytolyzed

(e) Oxygen Lack

Cyanide presumably acts by depriving the cells of oxidative energy through suppression of iron catalysis. Direct oxygen lack probably produces the same end results. Experiments at various oxygen tensions were not performed because pure gases for mixtures were not readily available. However, observations on eggs that were crowded may show something of the effects of partially anaerobic conditions.

When fertilized eggs were allowed to stand in sea water with more than several layers of eggs on the bottom of the dish, development was irregular. Some eggs would proceed at the normal rate while others would not divide at all. Since the eggs are large and respire actively this was very likely due to the local removal of oxygen from the water. (A much more concentrated suspension aerated by shaking in the manometer flasks showed normal development.) Only those eggs at the surface could obtain enough oxygen to undergo cleavage at the same rate as control eggs that were spread thinly on the bottom surface.

If eggs were piled up in a vial of sea water to form a layer 1 cm. deep, only those at the surface developed. The lower layers showed neither development
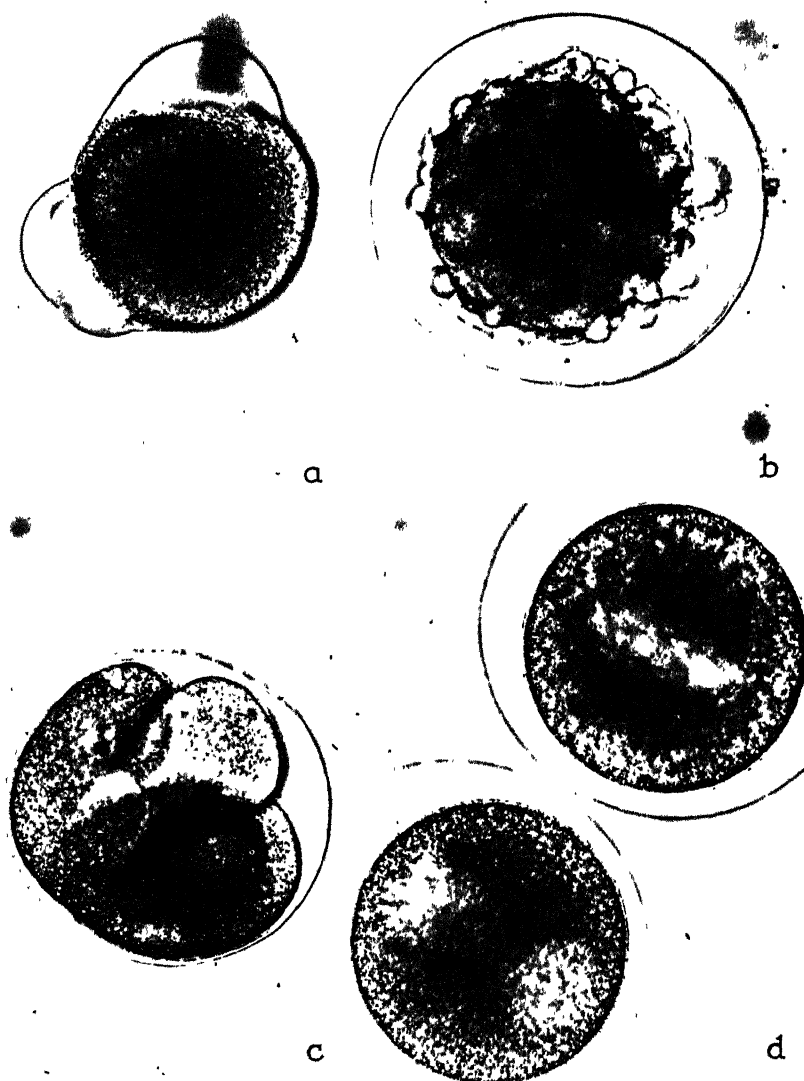
FIG. 11. Photomicrographs of *Echinarachnius parma* eggs in cyanide solutions. *a*, unfertilized egg after 4 hours in 10^{-4} M HCN, showing appearance of blebs. *b*, fertilized egg, same conditions as *a*. *c*, fertilized egg after 4 hours in 10^{-6} M HCN; cell division has been attempted but the blastomeres are incompletely separated. *d*, fertilized eggs after 5 hours in 10^{-2} M HCN. (Eggs placed in cyanide 8 minutes before the control group started to cleave.) Cell division has been completely arrested and there is no cytolysis.

or cytolysis for 2 days.   A sample of eggs removed from the center of the mass after 1 day and placed in a large volume of sea water cytolyzed completely within a few hours.   Possibly the cytolytic breakdown itself is dependent on an oxidative process, and either complete lack of oxygen or a high concentration of cyanide (10^{-2} M) blocks it for this reason.

DISCUSSION

The preceeding experimental data show the dependence of the *Echinarachnius* egg on aerobic metabolism for both cleavage and maintenance. The close correlation between the HCN concentration curves for inhibition of respiration and of cell division demonstrates the former point, and the observations on cytolysis and lethal dosage are evidence of the latter.

It is possible to classify marine eggs according to their respiratory requirements into three groups: (1) those that require respiratory energy for both maintenance and development; (2) those that will survive prolonged periods of anoxia but must respire in order to carry on cell division; and (3) those that can both live and develop under relatively anaerobic conditions. *Echinarachnius* is an excellent example of group one, *Arbacia* of group two, and *Fundulus* of the third group. As might be expected the sensitivity of these eggs to cyanide parallels their response to oxygen lack; the sand dollar is killed by short exposures to concentrations of cyanide that inhibit oxygen consumption; development of *Arbacia* is prevented when the oxygen uptake is lowered by cyanide, but the eggs will survive prolonged respiratory depression with little loss in developmental capacities; the *Fundulus* egg which is heavy and sinks into the bottom mud during its development will show normal cleavage in cyanide solutions of high concentration.

The maintenance of normal cellular structure and the operation of such functional processes as cleavage call for a certain output of energy.   If respiratory energy release is blocked by cyanide the cell requires another source of energy for survival.   In the *Arbacia* egg this need may be met by a glycolytic mechanism, since it has been shown that lactic acid is produced during cyanide exposure (13).   The inability of *Echinarachnius* to survive HCN inhibition of respiration suggests an almost complete dependence upon an aerobic energy system.

Variation in the sensitivity of the eggs to cyanide at different stages of development is possibly a reflection of different maintenance energy requirements.   Unfertilized eggs in a "resting" state may maintain normal structure and developmental potentialities at a level of energy release which is grossly inadequate to support actively developing individuals.

SUMMARY

Cyanide is a valuable tool for studying respiratory mechanisms and their rôle in embryonic development: it is relatively specific in its action, penetrates

cell membranes readily, is active in low concentration, and may be controlled quantitatively (page 217).

Echinarachnius is extremely sensitive to cyanide and the oxygen consumption of both eggs and of sperm is almost completely inhibited by 10^{-5} M HCN (pages 219 and 221). Cell division is likewise arrested by the same concentration (page 223).

One of the pronounced effects of an irreversible dosage of cyanide is the marked cytolysis or breakdown of the egg, both internally and at the cell membrane. This cytolysis appears to be related to the state of metabolism, and its occurrence varies with both the respiratory and developmental activity of the cell (page 224).

The lethal dosage of cyanide varies with the state of development of the egg: the unfertilized egg is less susceptible than the fertilized one, and the susceptibility increases as the development of the fertilized egg proceeds (page 228).

The *Echinarachnius* egg differs from that of *Arbacia* in respiratory behavior chiefly in its inability to survive prolonged anoxia: the sea urchin egg will tolerate for 24 hours a concentration of cyanide that kills the sand dollar eggs in 30 minutes (page 229).

The *Echinarachnius* egg is apparently completely dependent upon cyanide-sensitive catalytic systems for its normal functioning and maintenance. Interference with this aerobic energy release mechanism results in irreversible damage to the egg (page 231).

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PHAGE FORMATION IN STAPHYLOCOCCUS MUSCAE CULTURES

I. A Factor Necessary for Phage Formation

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It has been reported previously (Price, 1947 *a*) that the phage for *Staphylococcus muscae* will not increase in the synthetic medium of Fildes (Fildes and Richardson, 1937), although this medium supported bacterial growth. The addition of yeast extract or veal infusion broth to the synthetic medium permitted large increases in phage.

In this paper it will be shown that the substance necessary for phage formation, under the above conditions, is present in acid digests of highly purified proteins.

Methods

The same synthetic media test system was used as described previously (Price, 1947 *a*). All phage dilutions were made in distilled water. The bacteria, grown as described previously (Price, 1947 *b*), were centrifuged, washed once with saline, recentrifuged, and suspended in distilled water. 0.1 ml. of bacterial suspension was added to each reaction mixture. Bacteria and phage were determined as described previously (Price, 1947 *b*).

Assay for Factor.—The reaction mixture consisted of the following: the synthetic medium of Fildes (6.0 ml. of the amino acid mixture, 0.25 ml. of M/2 glucose, 0.25 ml. of M/500 ferrous ammonium sulfate, 0.10 ml. of vitamin B_1 which contains 100 γ of the vitamin, 0.10 ml. of nicotinic acid containing 100 γ of the vitamin, 0.10 ml. of M/60 $MgSO_4$, 0.10 ml. of M/5 sodium nitrate, 0.20 ml. of 1/100 M cystine, 0.10 ml. of M/1000 tryptophane, 0.20 ml. of M/100 methionine, 0.20 ml. containing 800 γ of oxyproline). To this mixture was added 0.1 ml. of bacteria suspension to give a final concentration of 1×10^8 organisms per ml., and 0.5 ml. of a phage solution containing 1×10^5 phage particles. The reaction mixture was then made up to 10.0 ml. with H_2O. 0.25 ml. of the material to be assayed was then added. All samples were shaken at 36° as described previously (Price, 1947 *b*). Under the above conditions, complete lysis of the system took place within 6 hours when sufficent phage-promoting substance was present. Complete lysis of the system was used to assay for the factor and was a reproducible sign of phage growth. However, as Table I shows, small concentrations of the factor can cause large increases of phage without lysis. This phenomenon will be discussed in the next paper of this series. Phage determinations were carried out

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when the addition of 25.0 mg. of protein did not cause lysis, but in all such cases no increase in phage was found.

Sources of Protein.—Dr. M. Kunitz kindly supplied 3 times recrystallized chymotrypsinogen, 4 times recrystallized trypsin, 7 times recrystallized chymotrypsinogen, 4 times recrystallized ribonuclease, 4 times recrystallized No. 2 protein from yeast (Kunitz and McDonald, 1946), and 3 times recrystallized No. 3 protein from yeast (Kunitz and McDonald, 1946). Dr. R. M. Herriott generously furnished 2 times crystallized edestin, 5 times recrystallized egg albumen, and crystalline pepsin. Dr. W. M. Stanley kindly furnished crystalline tobacco mosaic virus. Crystalline insulin was obtained from the Eli Lilly Company. Dr. E. Cohn of Harvard University kindly supplied the serum albumen and serum globulin.

The experiments with casein were carried out with casein, Hammarsten, procured from Amend Drug and Chemical Company, vitamin-free casein digest from General Biochemicals Inc.

TABLE I

Effect of Various Concentrations of Hydrolyzed Casein on Phage Formation

Each sample contained the usual synthetic medium. Hydrolyzed casein was from the General Biochemicals Inc. Sample for phage assay taken at end of 6 hours.

Sample	Hydro-lyzed casein	Plaque count per ml.		Cell count per ml.		
		Initial	Final	Initial	Maximum	Final
	mg.					
1	—	5.6×10^5	2.1×10^5	1.3×10^8	5.3×10^8	5.3×10^8
2	1.0	5.6×10^5	6.1×10^{10}	1.3×10^8	5.2×10^8	5.2×10^8
3	15.0	5.6×10^5	1.1×10^{10}	1.3×10^8	3.7×10^8	0

RESULTS

The factor necessary for phage production was present in acid digests of vitamin-free casein. In view of this finding, the presence of the factor in other proteins was investigated. Table II shows the presence or absence of this factor in fifteen proteins. Casein has the highest concentration of factor with pepsin having the next greatest concentration (Table III). Ribonuclease, insulin, tobacco mosaic virus, and the No. 2 protein from yeast do not have any factor in concentrations of protein as high as 75 mg. per 10.0 ml. of reaction mixture.

Unhydrolyzed proteins have no phage-promoting activity. One time crystallized chymotrypsinogen has a slightly lower activity than 8 times recrystallized chymotrypsinogen. These experiments indicate that the factor is not adsorbed to the protein, but is an integral part of the molecule.

Release of Factor by Acids.—The factor is formed from proteins by treating them with 3 N HCl or 3 N H_2SO_4 for 1 hour at 15 pounds pressure. Longer hydrolysis does not release more of the factor. It is not formed by concentra-

tions of NaOH up to 3 N.   The factor was not destroyed under the latter conditions, since treating the inactive alkali digest of casein with 3 N HCl released

TABLE II

Test for Factor in Acid Digests of Purified Proteins

100 mg. of each protein was hydrolyzed in 1.0 ml. of 3 N HCl for 1 hour at 15 pounds pressure.   0.25 ml. of each hydrolysate was added to the usual synthetic mixture.   The pH of the mixture was then readjusted to pH 7.6 with N NaOH.   Conditions as described under Methods.

Active	Inactive
Crystalline chymotrypsinogen	Crystalline insulin
" chymotrypsin	" tobacco mosaic virus
" trypsin	" ribonuclease
" pepsin	" No. 2 yeast protein
Serum albumen	
" globulin	
Crystalline egg albumen	
" edestin	
" cucumber seed globulin	
" No. 3 yeast protein	
" trypsinogen	
Gelatin	

TABLE III

Concentration of Factor in Proteins

Proteins were hydrolyzed as in Table I.   All proteins were added in quantities of 25 mg., 20 mg., 15 mg., 10 mg., 5 mg., 2 mg., and 0.750 mg.   Listed below is that quantity which caused complete lysis of the system.   Conditions described under Methods.

Protein	Quantity used
	mg.
Casein	4
Pepsin	6
Edestin	10
Chymotrypsinogen	15
Gelatin	20
Egg albumen	20
No. 3 yeast protein	25

the factor.   Active acid digests of casein treated for 1 hour at 15 pounds pressure with 3 N NaOH are not destroyed.   The substance is stable after refluxing in 6 N HCl for 48 hours.

Factor Released by Enzymes.—The factor is not formed from casein by pepsin, trypsin, chymotrypsin, or the combined proteolytic activity of the three en-

zymes. The substance is released from egg albumen and pepsin by peptic action, but not by trypsin, chymotrypsin, or the combined action of the latter two enzymes. Treating an egg albumen peptic digest with 3 N HCl at 15 pounds pressure for 1 hour does not release any more of the factor than was released by the action of pepsin (Table IV).

Addition of Amino Acids.—Cysteine, serine, threonine, isoleucine, and norleucine, in a concentration of 40 γ per ml. of reaction mixture, do not have any

TABLE IV

Release of Factor by Enzymes

All enzymatic digests carried out in 125 ml. flasks under toluol at 40° for 48 hours. 500 mg. of each protein in a volume of 50 ml. was used for enzymatic digestion and 5 mg. of enzyme. Pepsin digestion was carried out at pH 2.0 and trypsin and chymotrypsin digestion in 0.1 M phosphate buffer pH 7.6. On testing action of combined proteolytic enzymes the casein was first digested with pepsin for 48 hours and then pH readjusted and digested with trypsin for 48 hours, followed by chymotrypsin for 48 hours. In the digestion of egg albumen and pepsin by trypsin-chymotrypsin, trypsin was added first followed by chymotrypsin. Controls were run containing the proteins without the enzymes and the enzymes without the proteins at the various pH at 40°C. under toluol. 25 mg. of each protein digest was used for each test. Enzymatic digestion carried out at 5°C. for 3 weeks gave the same results as digestion at 40°. Conditions of test as described under Methods.

Active digests	Inactive digests
Pepsin digest of pepsin " " " egg albumen	Pepsin digest of casein Trypsin " " " Chymotrypsin digest of casein Pepsin-trypsin-chymotrypsin digest of casein Trypsin and chymotrypsin digest of egg albumen or pepsin Trypsin-chymotrypsin digest of egg albumen or pepsin

phage-promoting activity under the experimental conditions. The medium already contains 16 amino acids.

Test for Factor in Phage and Medium.—Bacteriophage, purified by centrifugation, was hydrolyzed to determine whether the factor was incorporated into the phage molecule without undergoing any change. The medium in which the phage grew was also tested for the presence of the factor. From Table V it can be seen that there was no factor present in the phage or lysate after acid hydrolysis even though a great excess of both substances was tested.

DISCUSSION

A factor necessary for the formation of *Staphylococcus muscae* phage has been found in acid digests of many highly purified proteins. The evidence indicates

that the active substance is not adsorbed on the proteins, but is an integral part of the molecule. The substance does not appear to be a peptide, as it is very stable in strong acid and alkali. Although twenty-one amino acids were tried, they could not replace the phage-promoting activity of the acid hydrolysates of proteins.

TABLE V

Test for Factor in Phage and Lysate

5,000 ml. of synthetic medium was set up containing 4.0 mg. of hydrolyzed casein per 10.0 ml. of reaction mixture, 3.3×10^8 cells per ml., and 3.5×10^5 phage particles per ml. Complete lysis took place at 6 hours. The phage count had risen to 2.1×10^{10} particles per per ml. The lysate was centrifuged for 1 hour at 14,000 R.P.M. The precipitate, which contained all the phage activity, had 76.3 mg. of protein. It was suspended in 1.0 ml. of 3 N HCl and hydrolyzed as described earlier. 1,000 ml. of the supernatant fluid of the centrifuged lysate was evaporated to dryness* on a steam bath and dissolved in 5.0 ml. of 3 N HCl and hydrolyzed as described previously. To another 1000.0 ml. of the supernatant fluid was added 10.0 mg. of hydrolyzed casein. This solution was then evaporated to dryness on a water bath, dissolved in 5.0 ml. of 3 N HCl, and hydrolyzed in the usual manner. 1.0 ml. of this sample was added to sample 4. Three mg. of hydrolyzed casein was added to a centrifuged phage preparation prepared from 700 ml. of synthetic medium which contained 4.1 $\times 10^{10}$ particles per ml. after complete lysis had taken place. The phage and hydrolyzed casein solution was then hydrolyzed in the usual manner in 0.5 ml. of 3 N HCl. 0.25 ml. of the hydrolysate was added to sample 5. These latter two tests show that the substance could have been detected by the method used. The test conditions are described under Methods. Assay for phage made at end of 6 hours.

Sample	Additions	Phage count per ml.	
		Initial	Final
1	0.8 ml. of hydrolyzed phage	3.1×10^5	6.1×10^5
2	1.0 " " " lysate	3.1×10^5	8.3×10^5
3	1.0 mg. " " casein	3.1×10^5	1.8×10^{10}
4	1.0 ml. " " lysate plus added casein	3.1×10^5	2.3×10^{10}
5	0.25 " " " phage plus added casein	3.1×10^5	1.7×10^{10}

*All samples were extracted with hot butyl alcohol, pH 5.5, and then evaporated to dryness before being dissolved in water.

The factor undergoes a change when added to a multiplying bacteria-phage system, since neither purified phage nor the concentrated supernatant fluid from a centrifuged lysate shows any phage-promoting activity when hydrolyzed with acid in the usual manner. From the results at the present time, therefore, it is not possible to decide whether the factor is incorporated into the phage or whether it is necessary as a "coenzyme" for a reaction which is essential in the synthesis of phage.

From the following results it appears that the synthesis of *S. muscae* phage is intimately bound to the metabolism of the host, (1) nicotinic acid is essential for phage formation and is utilized by the cell (Price, 1947 *a*); (2) the factor de-

scribed in this paper is also utilized by the cell (Price, 1947 a) and appears to be changed when added to a multiplying bacteria-phage system; (3) the synthesis of ATP by the cell seems to be essential for phage formation (Price, 1947 c). It also appears that the phage directs its own synthesis since niacin, the unknown factor, and phage must all be added at the same time to the medium if a synthesis of phage is to occur (Price, 1947 a). Once the two factors are taken up by the bacterial cell in the absence of phage, there is no increase in phage when the bacterial virus is then added (Price, 1947 a). If the phage was formed from a precursor, it would be expected that phage formation would occur even though the phage was added to the system after all the other essential components.

SUMMARY

1. A factor necessary for the formation of *Staphylococcus muscae* phage was found in acid digests of many highly purified proteins.

2. The factor is released from egg albumen and pepsin by peptic digestion.

3. No amino acids tried could replace the acid digests of proteins as a source of the factor.

4. The factor, when added to a multiplying bacteria-phage system, cannot be found in purified phage or in the lysate after complete lysis of the system has taken place.

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A VERSATILE MICRORESPIROMETER FOR ROUTINE USE*

By HERBERT STERN‡ AND PAUL L. KIRK

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Investigations under way in this laboratory required a microrespirometer which could be used for a considerable period of time in one set of measurements, and which also avoided some of the difficulties of manipulation encountered with previous models (Cunningham and Kirk, 1940; Barth and Kirk, 1942). Accordingly, an instrument was designed and built which had the following advantages as compared with earlier models: (1) It was more rapidly and conveniently assembled without leakage; (2) it could be employed for measurements over a longer time without opening; and (3) less difficulty was encountered in adjusting the indicator droplet.

In addition, it was designed to allow operation with controlled gas mixtures and mixing of reagents after sealing. These were features of the one earlier model (Cunningham and Kirk, 1940) which were not present in the simplified form described later (Barth and Kirk, 1942). The basic pattern of the instrument is similar to that of Barth and Kirk (1942), the chief deviations from that design being a different method of sealing, a considerably longer capillary measuring tube, and the installation of needle valves which allow both the easy adjustment of the indicator droplet and replacement of the gas in the chambers with a controlled mixture.

Description

The instrument is shown diagramatically in Fig. 1 and its assembled appearance in Fig. 2. The differential chambers were drilled in a block of duralumin $3\frac{1}{4} \times 1\frac{1}{2} \times 1\frac{1}{2}$ inches, which was considerably larger than the blocks of brass used by Barth and Kirk. To prevent reaction of the block with caustic in the event of spillage, the chambers were lined with a thin coat of lacquer. Into each chamber was drilled a hole through the side of the block, and in it was inserted a small needle valve. This allowed the chambers to be exposed so that the indicator bubble could be easily adjusted with respect to the scale just prior to measurement. The needle valves were constructed from steel.

The top plate was made of brass, $3\frac{1}{4} \times 1 \times \frac{3}{8}$ inches, which was considerably thicker than the plates previously used. By increasing the thickness, the
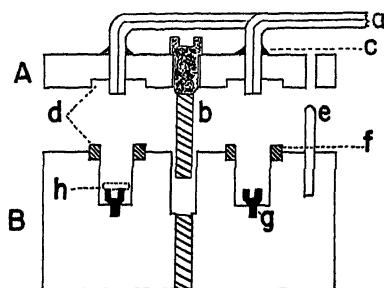
FIG. 1. Cross-section of microrespirometer (actual size). *A*, top plate; *B*, dura-lumin block; *a*, capillary tube; *b*, Allen screw; *c*, de Khotinsky cement; *d*, counter-sinks for housing rubber gaskets; *e*, supporting pin; *f*, rubber gasket; *g*, teflon cup; *h*, position of anther.
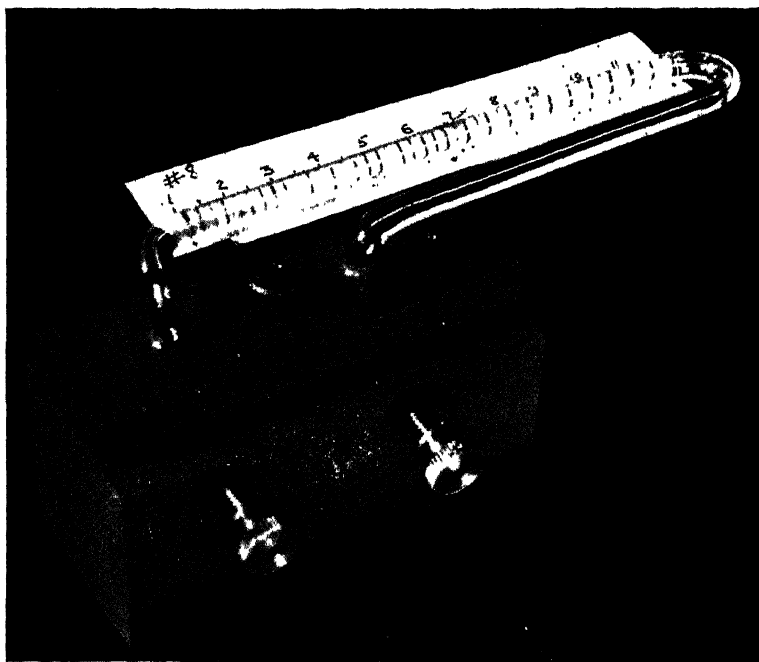
FIG. 2. Assembled microrespirometer.

entire plate could be clamped solidly by means of a single Allen type screw. The plate was sealed to each chamber by means of a firm rubber gasket, $\frac{3}{16}$ of an inch thick. The capillary which was sealed to the plate by means of de Khotinsky cement was bent in the form shown in order to increase the working

range of the respirometer. In bending it, great care was necessary to avoid constrictions of the bore which tended to trap some of the kerosene, thus preventing proper functioning of the respirometer. The diameters of the capillaries used in the present experiments were 0.3 to 0.6 mm.

With the exception of the differences discussed, the design and construction were very similar to those described by Barth and Kirk.

Operation

Before using a new respirometer it was necessary to clean the capillary bore scrupulously. This was done by treating it overnight in sulfuric-chromic acid mixture followed by several washings with distilled water and drying in dust-free air. Once cleaned, the capillary required little further attention.

Kerosene for the index droplet was purified by mixing it for several days with concentrated sulfuric acid, neutralizing with sodium hydroxide, dehydrating with anhydrous sodium sulfate, and distilling, with retention of the middle fraction only. The purified material was stored over pellets of sodium hydroxide.

Small cups of polytetrafluoroethylene (teflon) were used to contain about 50 λ of 1 per cent sodium hydroxide solution used for absorption of carbon dioxide. The arrangement is, however, arbitrary and depends largely on the type of tissue being used. In our case, the respiration of *Trillium* anthers was being studied so that small cups were convenient since the anthers could be rested on their rims. A piece of filter paper wet with distilled water was added to prevent drying of the tissue. The plate, carrying the capillary which contained an index droplet of purified kerosene, was put in place and tightened on the rubber gaskets with an Allen wrench. The droplet was shifted to the desired position on the scale by tilting the respirometer after which the needle valves were closed. The respirometer was placed in a simple air thermostat to maintain a reasonably constant working temperature. Small fluctuations in temperature which were not localized did not affect the measurements because of the differential principle used. The larger capillary index tubes, however, were susceptible to the effects of unsymmetrical exposure to radiation causing erratic movements of the kerosene droplet. To prevent this effect, a fluorescent light source was used and unnecessary exposure of the tubes to radiation was avoided.

With this design of respirometer, no more than 3 minutes were required to prepare it for use. Once charged with caustic, at least three successive determinations could be made merely by changing the anthers. With older models, the kerosene droplet was frequently lost in opening and closing the instrument. In this model the presence of needle valves was, therefore, of decided advantage. Clamping of the top plate caused no disturbance of the droplet since both chambers were still exposed to the atmosphere and the closing

of the needle valves affected the position of the droplet but slightly.   Only on opening of the valves did a rapid shift of the droplet occur, but this could often be avoided by opening both valves simultaneously; and when the droplet did break, it could be readily reconstituted by gentle blowing through one valve. Thus, in 500 determinations, kerosene had to be added only twice.

The data obtained by use of this respirometer are described in an accompanying paper.   About 3 to 4 mg. of plant tissue appeared to be the lower limit in mass of tissue necessary for good results.   With smaller amounts of tissue, it became necessary to check carefully for erratic drifting of the indicator droplet due largely to a vaporization of some of the kerosene.   It also appears advisable in such cases to use shorter capillary tubes and thus reduce the probability of their unequal heating or cooling.

Various adaptations of the instrument are possible and some minor improvements are at present being considered in manufacture.   The needle valves may be used for introducing gases or their mixtures into the chambers.   This is accomplished by lifting the top plates very slightly to provide an outlet for the gases which are introduced through the valves.   The operation is a little delicate and must be carefully controlled, since the rates of gas flowing through each of the chambers, if unequal, cause a rapid shifting and frequently a loss of the indicator of the droplet.   Also, as in the Cunningham-Kirk type, solutions may be mixed in the chambers; bicarbonate and acid can be used in this way to standardize the respirometer.

SUMMARY

A new design of microrespirometer suitable for routine laboratory work has been described.

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THE OXYGEN CONSUMPTION OF THE MICROSPORES OF TRILLIUM IN RELATION TO THE MITOTIC CYCLE*

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Up to the present time, investigations of the oxygen consumption of dividing cells have centered either on proliferating tissues containing a comparatively high percentage of dividing cells, or on a variety of animal eggs, before and after fertilization. For several reasons, including the lack of tissues homogeneous with respect to the stage of division, the information thus obtained has been largely confined to the grosser aspects of respiratory behavior. To those changes more closely allied to the development of the mitotic cycle there exist only scanty references, although such information must certainly be fundamental. It is from this standpoint that the anthers of many plants provide excellent material for study, because, as previously indicated (Stern, 1946), the remarkably slow rate of division of the pollen mother cells and the microspores allows for a more exact determination of the changes occurring at successive stages in the development of the nuclear cycle.

Recently, Erickson (1947) made the first attempt to elucidate this type of problem by measuring the oxygen consumption of excised anthers of *Lilium* in Fenn microrespirometers. His results constitute a broad survey of the oxygen consumption of the anther during its entire development, and clearly suggest that marked changes occur at the time of division of the pollen mother cells and the microspores. Our approach, though largely similar to that of Erickson, differs somewhat in respect to the method of referring the Q_{O_2} values obtained. Rather than using time after planting, and length of flower bud as reference points, the oxygen uptake of the anthers was referred to the stage of division in the microspores as determined from acetocarmine smears. It is of interest that the results of this investigation confirm and supplement those of Erickson.

In order to correlate most advantageously the oxygen consumption of anthers with the stage of division of the microspores, the anthers were studied singly. Each anther was weighed, its oxygen consumption measured, and a smear preparation made to check the stage of division. This procedure not only allowed for corrections in those cases where anthers of the same bud were at different stages of development, as did occasionally occur, but it also provided

for a check on the degree of variability within a plant.   Since *Trillium* anthers
at the microspore stage weighed 4 to 10 mg., the use of the microrespirometer
previously described (Stern and Kirk, 1948) was of distinct advantage.

Measurements of Oxygen Uptake

The *Trillium erectum* (L) plants used in these experiments were obtained com-
mercially and kept throughout at 3°C.   This treatment appeared to have no inhibi-
tory effect on the development of the microsporangial tissue; in fact, in nature,
division takes place under a carpet of snow.   Respiratory measurements, however,
were made at 25°C., and in order to assure the tissues being at thermostat tempera-
ture, all plants were kept overnight at room temperature before being used.   Just
what the effects of this sudden change in temperature were, was not studied.   It

Fig. 1. Diagram of quartz helix balance.   For explanation see text.

appears nevertheless that rigid adherence to the procedure given yielded Q_{O_2} values
which truly represented, from a relative standpoint, the normal behavior of the
developing anther.

In every instance, the oxygen uptake of each of the six anthers of a bud was meas-
ured simultaneously in six microrespirometers.   Fifteen minutes were allowed for
equilibration, and readings were then made after 15 minutes and after 1 hour.   In
line with Erickson's observations, there was little change in the rate of oxygen con-
sumption for the duration of the experiment.   At the end of 1 hour, the anthers were
weighed and acetocarmine smears were prepared to determine the stage of division.

Weighing of Fresh Anthers

In order to obtain the correct fresh weight of anthers, it was necessary to weigh the
material rapidly to minimize the loss of moisture.   This was performed very con-
veniently by use of a quartz helix balance shown in Fig. 1.   The quartz fiber helix
was made from uniform fiber about 0.1 mm. in diameter, wound to a total length of
about 40 cm.   It was suspended inside a glass tube 30 mm. in outside diameter and

with a telescoping tube at the bottom, 34 mm. in outside diameter. A small quartz cradle served to support the anther. The elongation of the spring which is, with quartz helices, exactly proportional to the weight applied, was read with a simple cathetometer. Rapid weighings to 0.01 mg. were readily possible, since the spring elongated about 6 mm. per mg. The readings were independent of temperature fluctuations within narrow limits because of the extremely low coefficient of expansion of fused quartz.
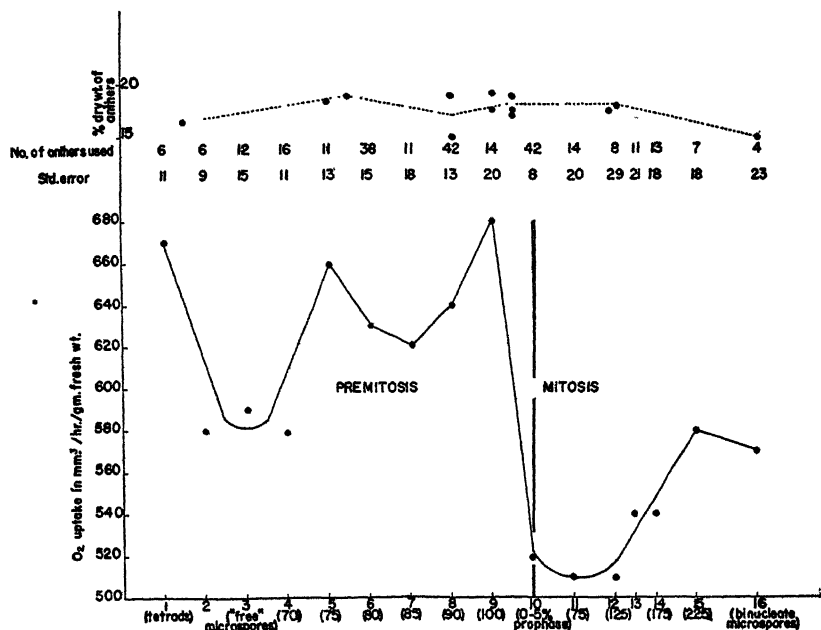
FIG. 2. The oxygen consumption of anthers during development of microspores. The bracketed numbers in the premitotic part of the scale indicate average diameter of microspores (arbitrary units); those bracketed in the mitotic part indicate the degree of development as explained in the text. The dotted line represents the changes in hydration of the anthers, each point having been obtained from studies of five anthers.

RESULTS

A total of 265 measurements of oxygen consumption of *Trillium erectum* anthers was made as described, and was plotted against the various successive stages in the morphological development of the microspores. The results are summarized in Fig. 2, in which the over-all trend appears to consist of a rise in the premitotic stages followed by a sharp drop prior to the onset of active division. It should be pointed out that the criteria by which the trend is established are not without some uncertainty. The stages in premitotic development, during which fluctuations in the curve are apparent, could not be clearly

defined.   Nevertheless, allowing for a margin of error, a fairly reliable if some-
what arbitrary scale may be set up.

Following meiosis, the quartet of microspores which are closely grouped in
"tetrads" begins to "loosen," and eventually the microspores appear to be no
longer clustered.   At this point they are comparatively small in size, but as they
mature towards active division they show an over-all increase in diameter.
By no means is any one anther characterized wholly by a single size of micro-
spore, but it is possible, quickly and without much difficulty, to estimate the
average.   Thus, plotted against microspore size, the Q_{O_2} values showed a pro-
gressive increase with development.   One lone peak in the premitotic portion
of the curve stands off from the general trend, but for the present, it may be dis-
regarded, and the premitotic trend considered as a steady rise in oxygen uptake.

The most pronounced change occurred in the anthers containing the micro-
spores of largest diameter.   Here, clearly, the rate of O_2 uptake in some anthers
was high, in others low.   It was further observed that those anthers charac-
terized by a low rate usually had a small percentage of microspores in active
division; if not so with all the anthers of the bud, then at least with some of
them.   It was therefore decided to segregate these anthers into two groups:
those in which none of the anthers in the bud showed any signs of active mito-
sis (stage IX), and those in which at least two of the anthers in the bud con-
tained actively dividing microspores (stage X).   The result of such a classi-
fication is apparent, and from it, it may easily be inferred that at some point
preceding active division there is a sharp drop in oxygen uptake of the anthers.

With an increase in the percentage of actively dividing microspores, there
appears to be no appreciable change in the rate of oxygen uptake.   This is
fortunate because translation of the data in terms of individual mitotic stages
is not simple.   At most times during active division, there is a distribution of
cells among some or all of the various stages of development.   The best that
can be done, therefore, is to estimate the degree of development by calculating
the relative proportions of the various stages present in the anther.

To express the development of the microspores in terms of percentage of
cells in active division would be unsatisfactory since that would leave out of
account the proportion of cells yet to divide and those already divided.   It is
much better to assign a series of increasing numbers, 0, 1, 2, and 3 to premitosis,
prophase, metaphase, anaphase, and binucleate stages respectively and to sum
the products of these numbers times the percentage frequency of the corre-
sponding stages in the preparation.   For example:

	Premi-tosis	Prophase	Meta-phase-ana-phase	Binucleate	Total
Frequency, *per cent*........	62	19	5	14	
Number × per cent.....	0	19	10	42	71 .

The use of these numbers, of course, is merely a convenience; they are not intended as a quantitative expression of development.

In general, however, the larger the proportion of cells in the more advanced categories of division, the larger the total number; and when Q_{O_2} values were plotted in this way, the behavior of the anthers, as the microspores proceeded to completion of mitosis, could be inferred.

<div align="center">DISCUSSION</div>

It may be assumed that during the period of measurement, there was little progress in the development of the microspores. The rate of mitosis is slow, for even at room temperature the transition from the premitotic to the binucleated microspore occupies at least 5 to 6 days. Obviously, the change during the interval of the experiment must be very small, and the Q_{O_2} values obtained may therefore be assumed to be representative of the stage of division as determined from the acetocarmine smears.

The values given represent the oxygen uptake of the whole anther, and it may well be asked to what extent the fluctuations observed represent the situation in the developing microspores. From twelve anthers tested, it was found that the microspores plus the antheral sap constituted approximately 35 per cent of the total fresh weight of the anther. The microspores must constitute even less. It would seem then that the magnitude of the changes observed is but a fraction of those which occur in the microspore, provided, of course, the changes are not due to the remainder of the anther. From many standpoints, changes other than those occurring in the microspore do not seem likely. The morphological development of the anther, microspores apart, is not characterized by marked changes corresponding to the respiratory ones described. Erickson, in fact, suggests a gradual diminution in rate of O_2 uptake with development, a general characteristic of maturation. On the other hand, the marked correlation of the inflexions in the curve here shown with the mitotic state of the microspores points emphatically to the microspores as the factors most responsible for the kind of behavior observed.

If it be assumed, then, that the changes are largely a reflection of the behavior of the microspores, some conclusions respecting mitotic development and oxygen uptake may be drawn. Two extremes of behavior are at once apparent: a comparatively high rate of oxygen uptake occurring some time before the onset of active division, and a comparatively low one beginning before the onset of active division and continuing through it until the termination of the cycle. In view of this, the oxygen requirements of cells in mitosis would seem to be a moot question. The conclusion, for example, that the positive correlation between high O_2 uptake and high mitotic frequency indicates a high consumption of oxygen during mitosis (Beatty, 1946) may be only partly right, for the values thus obtained may largely reflect the oxygen requirements immediately preceding active division. It would, of course, be hard to infer from

the data on microspore behavior just what constitutes "normal" oxygen up-take; most likely, both extremes lie on either side of such a value.

From the standpoint of speculation, the field is inviting—and wide open. It is hardly probable that energy requirements of cells should fall during division; indeed, the reverse is more likely so that oxygen uptake cannot here be properly regarded as indicative of respiratory activity. The immediate source of energy for active mitosis may not require molecular oxygen; in fact, the possibility of such behavior being universal is suggested by a comparison of these results with those of Rapkine (1931) on sea urchin eggs, which showed that aerobic oxidation may be inhibited without interfering with the progress of nuclear division. The problem, however, requires a more complete approach, and since more elaborate studies are at present being undertaken, further discussion is deferred in anticipation of a broader range of information.

SUMMARY

The oxygen consumption of 265 single *Trillium erectum* anthers was measured before and during the mitotic cycle of the microspores using a modified differential microrespirometer.

The results show a rising oxygen consumption of the anther in the premitotic stages followed by a sharp drop immediately preceding and during active division. It is suggested from these results that active division may be associated with anaerobic behavior and that the rapid uptake of molecular oxygen commonly associated with proliferating tissues is probably characteristic of premitotic development.

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SENSITIZATION TO HEAT BY X-RAYS[*]

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The possibility that sensitization to heat occurs following x-radiation has apparently not been reported. By sensitization to heat is meant a change induced by the radiation such that the cell is killed by a sublethal exposure to heat. When the heating precedes the exposure to radiation the cells are not killed by equivalent dosages. Since it has considerable theoretical interest as well as likely practical value, experiments were performed to test the possibility; the results were positive.

As experimental material *Paramecium caudatum* was used and the cultures were handled in the same manner as previously described (Giese and Crossman, 1945, 1946). The x-ray equipment used was designed by Dr. Harry Clark for the specific purpose of delivering high intensities of soft radiations; the apparatus has been described before (Taylor, Thomas, and Brown, 1933; Brown, 1933). In the present study it was run at 20,000 volts and 30 milliamperes and a silver target was used. The radiations were filtered through a 0.05 mm. thick aluminum window which completely absorbs the visible as well as the heat rays which are conducted to the water-cooled parts of the tube. The target and filament were rotated 45° for measurement of the dosage rate with an ionization chamber and a determination was made before or after each day's series of experiments. The dosage rate is of the order of 56,000 roentgens per minute. The average wave length is about 1 Å. The exposure cell was a celluloid container about 30 mm. in diameter and 4 mm. high. A drop of the culture of approximately the same size each time was placed in the center of the dish at the point of focus of the radiations.

The dosage of the radiations as given in the test must be corrected by a factor for their absorption by the medium. Thus Brown has calculated that 0.1 inch of water will remove 65 per cent of the soft radiations delivered by the tube. A protozoan at the bottom of a drop might receive only ⅓ of the radiation of one at the top (the x-rays being delivered from the top). During the course of exposure the paramecia swim throughout the drop, and except in dosages running over 400,000 r they are not markedly slowed; they come to rest at the bottom of the drop only after very large dosages.[1] In most cases the dosage at about the middle of the drop is probably

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[1] After dosages of 280,000 to 410,000 r the paramecia are especially sticky so that it is difficult to handle them. In 15 to 30 minutes after irradiation they tend to stick to one another to form clumps which become rather inactive. Later they again redisperse.

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an average dosage. This would be of the order of one-half of the incident dosage, but since it cannot be calculated with certainty it seemed better to give all the data as incident dosage. It must be borne in mind that the correction for very large dosages is greater than for the small and intermediate dosages.

<center>EXPERIMENTAL</center>

Before proceeding with the experiments on the sensitization to heat, paramecia were first exposed to x-rays to determine the lethal dosage with the particular radiations and stock to serve as a basis of comparison. The lethal dosage was found to depend upon the age of the culture used in the experiment. Thus paramecia in the logarithmic phase of a culture (first 2 days) are much more sensitive and variable than those in the stationary phase (4th to 7th day), therefore most of the data were gathered for the latter phase. During the stationary phase the paramecia are relatively free of food vacuoles and rarely divide. After a dosage of 1,200,000 r delivered in 20 minutes the paramecia were already dead and the majority had cytolyzed; the remainder cytolized soon afterwards. Given a dosage of 840,000 r they were still alive but died within 15 minutes after irradiation. After a dosage of 560,000 r they were alive but within 25 minutes about 10 per cent had died, in 50 minutes 80 per cent, and after 75 minutes all were dead and most had cytolyzed. After a dosage of 420,000 r they survived but did not divide for a long time. In contrast to this, paramecia taken from cultures in the 2nd day after inoculation (logarithmic phase) were killed by a dosage of as little as 140,000 r.

The above results are in agreement with the rule of Bergonie and Tribondeau (1906) which states that the sensitivity of cells is directly related to their activity. Since such active cells are not only sensitive to x-rays but also to heat (Doudoroff, 1936) and killing with heat occurs too rapidly for effective measurement of sensitization, paramecia in the stationary phase of a culture were used in all of the subsequent experiments unless otherwise indicated.

While these dosages may seem enormous, protozoa are notably resistant to x-rays (see Scott, 1937, for comparative studies). Taylor, Thomas, and Brown (1933) employing the same apparatus as in our studies found that *Euplotes* died in 15 minutes after a dosage of 450,000 r. Back and Halberstaedter (1945) found the lethal dosage for paramecium to be of the order of 700,000 r and Halberstaedter and Back (1942) found that between 300,000 and 600,000 r was lethal to *Pandorina*. If the correction for absorption is made to our data, the order of magnitude is similar to that found by other investigators.

Taylor, Thomas, and Brown (1933) found that when sterile medium alone is irradiated and the protozoa are added to it, death may occur. They conclude that death is due to development in the medium of toxic substances among the chief of which is probably hydrogen peroxide. In fact the dosage required to make the medium lethally toxic was only slightly greater than the dosage required to kill the animals directly. However, they found that organic materials

such as sheep's blood, gelatin, agar, and bacteria protected the protozoa against peroxide. In the present experiments it was observed that 1,000,000 r did not render the medium so toxic as to kill the introduced animals yet it was more than a lethal dose if applied directly. It is likely that in the present experiments the paramecia are protected from such concentrations of peroxide as are produced, by the bacteria and the organic materials present in the lettuce infusion in which they are suspended. On the other hand if the paramecia are removed from the medium in which they are irradiated and placed in fresh medium immediately after exposure, they are much less readily killed. This is true whether pond water or lettuce infusion with bacteria is used; the latter is especially effective. Thus 560,000 r never killed the paramecia from the stationary phase and even 840,000 r killed only about one-half, 1,000,000 killed a majority, and 1,200,000 killed almost all. In the latter case most of the protozoans are dead when they are removed from the radiation chamber and may therefore have already been affected by the poisonous material in the medium. The paramecia which survive the huge dosages do not divide for several days even though living in the midst of the bacteria which serve as excellent food for the controls.

Heat Sensitization

If paramecia irradiated with a sublethal dosage of x-rays are subjected to a sublethal heat exposure they succumb. Thus a dosage of 140,000 r does not injure paramecia noticeably, except to slightly retard division but if the animals so exposed are subjected to 1.5 minutes of heat at 42°C. they die. If the experiment is carried out in reverse, that is the paramecia are heated for 1.5 minutes to 2 minutes and then irradiated with 140,000 r they do not die. They become sluggish as do paramecia heated for the same length of time, but they remain alive and become more active again after the lapse of a few hours.

The data for a typical experimental series are plotted in Fig. 1. Thus as shown in one of the experiments 56,000, 140,000, and 280,000 r will sensitize paramecia to heat of 42°C. to such an extent that they will die within 3, 1.5, and 0.5 minutes, respectively, whereas the control requires about 6 minutes' exposure under the same conditions. It is apparent that the phenomenon is similar to that already described for ultraviolet light and for photodynamic action (Giese and Crossman, 1945, 1946).

Cells from the logarithmic phase of a culture are even more sensitive to heat than those from the stationary phase as already stated. It would be interesting to know whether such paramecia can be sensitized. Even though accurate measurements cannot be made with such animals owing to their rapid death following x-raying or heating alone, it was possible to demonstrate that they are sensitized as shown[2] in the data of Fig. 1.

[2] It is probable that logarithmic phase animals are sensitized to even lower temperatures than those from the stationary phase. Such experiments were not tried.
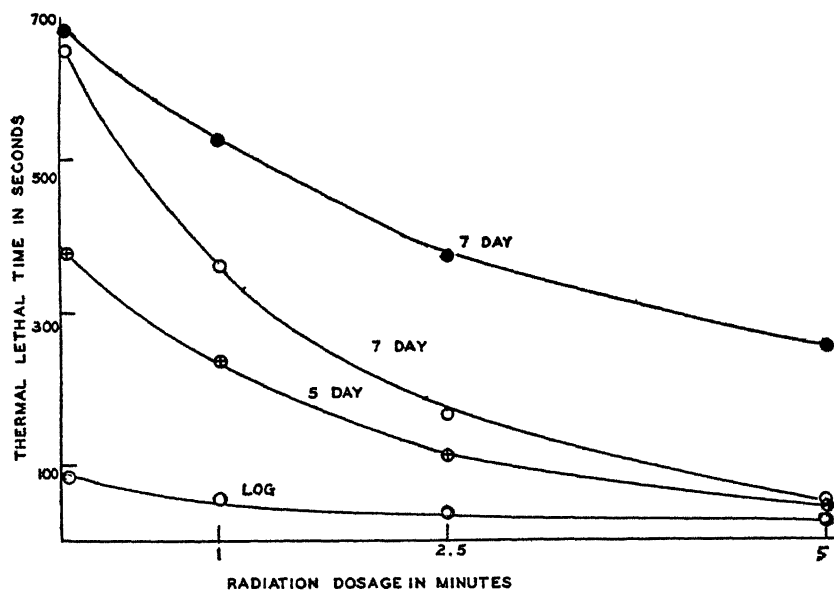
FIG. 1. Sensitization to heat by x-rays. The exposure in roentgens has to be corrected for the absorption by the medium as explained in the text. The stationary phase cultures are of different ages, the lowest being a 5 day, the upper two 7 day cultures. Why the top culture behaved as it did is not understood, but the results are rather variable.
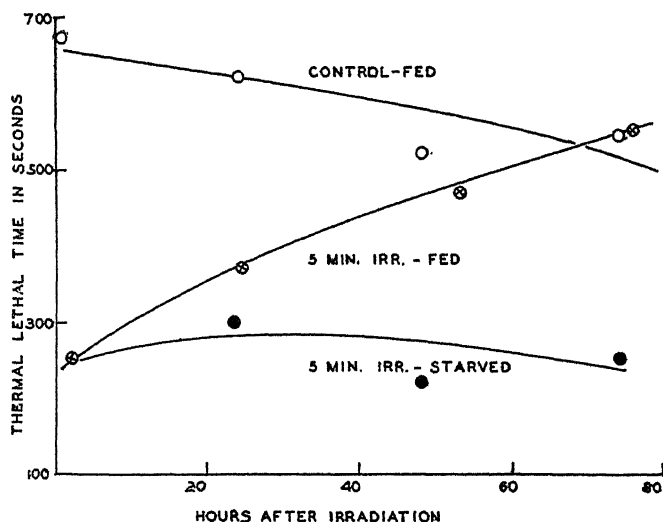
FIG. 2. Recovery from heat sensitization, incident dosage 280,000 r. Animals from the stationary phase of a culture used.

Recovery from X-Ray Effects

To determine the rate of recovery of paramecia from sensitization to heat, cultures were irradiated and samples were withdrawn periodically and tested. As shown in Fig. 2 there is no recovery if the paramecia are starved. When they are fed minimally—just enough to give a small percentage of divisions— they recover. It is difficult to add the exact amount of food which just allows recovery yet does not bring the animals into the logarithmic state in which a far greater sensitivity to heat is observed. But the results consistently demonstrated that recovery can occur and that it requires several days. If food is also withheld from the control the resistance slowly falls (Fig. 2).
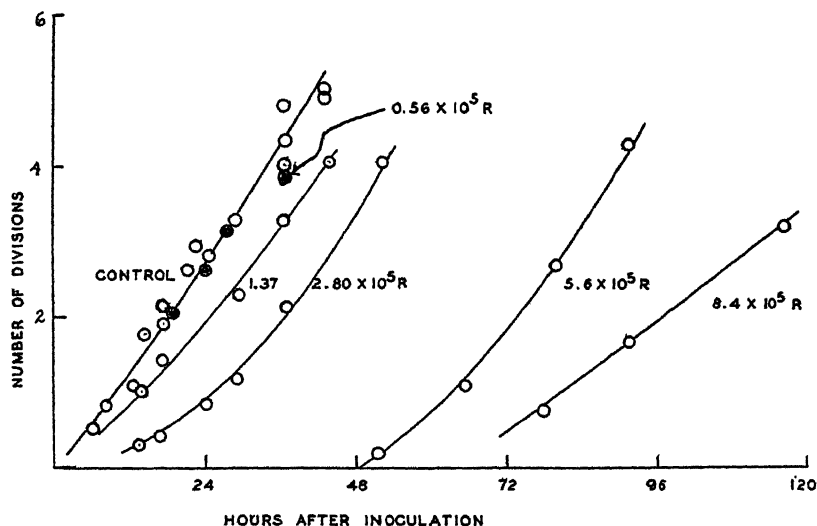
FIG. 3. Effect of x-rays on division. Dosages have to be corrected by the absorp. tion factor as explained in the text; real dosage about half or less than indicated-Experimental animals from the stationary phase of a culture.

If completely adequate food is supplied the paramecia divide as shown in Fig. 3. It is observed that a dosage of 56,000 r in no way alters the division for not only is there no observable lag but also there is no change in rate of division. After 137,000 r there is a pronounced lag; after 280,000 r this is increased and becomes quite marked after 560,000 r. But even with this very large dosage the rate of division, once division starts, is not altered since the slope of the curves is about the same in all these cases. But after a dosage of 840,000 r not only is there a prolonged lag but also a change in the rate of division.[3]

[3] The medium can be rendered toxic to paramecia so that division is retarded but very great dosages are required. A onefold dilution of such medium renders its

Paramecia transferred to culture medium containing an adequate supply of bacteria ordinarily become filled with food vacuoles, but irradiated paramecia may or may not take up bacteria, especially after large dosages of radiations. In such cases they may remain thin and free of food vacuoles for several days. It may be that in some way the x-ray injury prevents ingestion without which recovery and division cannot occur.

DISCUSSION

The experiments on sensitization to heat demonstrate that even a small dosage of x-rays which has neither visible nor division-retarding effects upon paramecia affects the protoplasm. In some way the resistance to heat is diminished. Nor is this effect a slight or temporary one for the recovery is quite slow. This has several implications, theoretical and practical, some of which will be considered below.

The slow recovery observed in our experiments contrasts with the results of Henshaw (1936) on unfertilized sea urchin eggs. Henshaw found that recovery was a matter of a few hours and from the graph relating it to the time elapsed, he concluded that it was due to the removal of some toxic material from the egg by diffusion outwards. He found that fertilization "fixed" the deleterious effects of the radiation so that recovery no longer occurred or did so very slowly. It is possible that certain processes which are going on in paramecium and which are affected by the radiations are not yet in full swing in the unfertilized egg and that when the activity metabolism (Fisher, Henry, and Low, 1941) is aroused by fertilization, catalysts needed for the processes so initiated may be poisoned by the toxic materials produced by the irradiation and so the rate of the reactions falls. Fisher, Henry, and Low have shown the activity metabolism to be susceptible to a number of respiratory poisons and furthermore have shown that the rate of division can be reduced and division entirely suppressed by critical concentrations of these poisons. Thus the conditions in the fertilized egg may correspond to those in paramecium and the difference is more apparent than real.

On the other hand perhaps a direct comparison between paramecia and eggs is impossible because of their different orders of sensitivity. Thus eggs of the West coast sea urchin, *Strongylocentrotus purpuratus*, were irradiated with the same apparatus as that used in the studies on paramecia and a dosage of only 6000 r strongly retarded division while a dosage of 14,000 r rendered the division completely abnormal. Such dosages are without effect upon paramecia

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toxicity negligible. Therefore, in the experiments cited in which the paramecia were removed to fresh medium immediately upon completion of the exposure this factor should not operate. In no case were monsters of any of the types described by Mottram (1942) seen.

and Packard (1933) has shown that successive dosages of 60,000 r could be given to paramecia within several hours without cumulative effects.

Two theories of the mechanism of action of ionizing radiations are current: (1) direct action of the particles or photons on the molecules (target theory) and (2) indirect action: radiations affecting the large molecules by activating water producing hydroxyl radicals, atomic hydrogen and hydrogen peroxide. The cytogenetic data support the target theory (see Lea, 1947) whereas most chemical effects of these radiations seem best explained by indirect action (Weiss, 1946; Barron, Dickman, and Singer, 1947) especially the work reported in the latter paper in which inactivation of sulfhydryl enzymes by ionizing radiations is shown to be reversed by addition of reducing compounds such as glutathione.
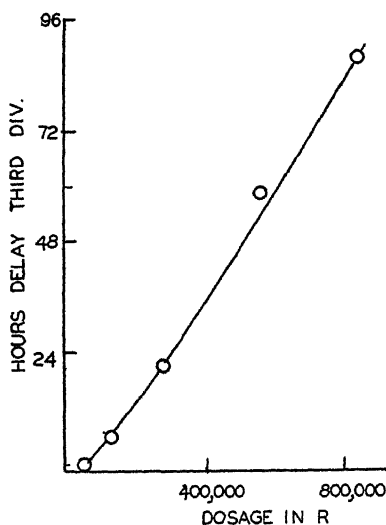
FIG. 4. Increase with dosage in delay of third division after raying. Note that the curve is practically a straight line.

Frequently recovery from physiological effects of ionizing radiations is rapid. In fact Lea (1947) interprets the convex curve obtained on plotting the dosage against the retardation in division of cells in tissue culture as an indication that recovery has occurred, otherwise a simple linear relationship might be expected. The present data, however, show that the lag in division of paramecia is proportional to dosage and the curve is practically linear (Fig. 4). This means that no recovery has occurred during this interval of time. The data suggest that the change in the molecules produced by the radiations is irreversible and recovery occurs slowly. Recovery probably requires the gradual replacement of the molecules altered. But even much smaller dosages than those which affect cell division markedly, none the less sensitize the cell to

heat and recovery from this sensitization is also slow. The fact that the cell recovers from heat sensitization only when it is supplied with food supports the conception of repair by replacement. It is interesting in this connection to note that animals in the logarithmic phase of a culture which are filled with food vacuoles show some recovery without being fed. The interpretation of radiation action by direct effects on the protein molecules also fits the data of heat sensitization as some sort of breakage or injury by x-rays of some bonds in protein molecules. If the animals are kept at room temperature the molecules so affected might continue to operate until replacement occurs. On the other hand at higher temperatures the molecules may break or the parts may become disoriented, leading to a loss of catalytic functions and death.

The possibility of practical application of the results on heat sensitization should not be overlooked even if the theoretical explanation is not clear. The effects of heat, diathermy, or short wave therapy applied concurrently, preceding, or subsequent to treatment with x-rays or radioactive elements, have been considered by various authors, some with negative, some with positive results (see Sugiura, 1941, for references to these papers). In the most recent work Sugiura takes advantage of the trials and difficulties of the earlier workers, and finds that x-rays sensitize tumor tissue to short wave radiation. Thus he showed that subsequent to a dosage of 500 r a regression of tumors of only 3 per cent occurred whereas among mice which in addition to x-raying were given a fever of 42°C. by short waves, 20 per cent of the tumors regressed; with 750 r the corresponding values were 15 per cent and 45 per cent. Although it is not entirely agreed upon by investigators using short waves that such radio waves act on cells only by virtue of their heating effects, it is probable that their main effect is just this (Kahler, Chalkley, and Voegtlin, 1929). The present experiments on paramecia therefore may possibly serve as a basis of explanation of Sugiura's findings. X-rays presumably sensitize the tumor cells to the heat developed by the radio waves. Since recovery from such sensitization requires several days, the repeated exposures to short waves are useful. The problem is certainly an interesting one for further investigation. If sensitization of tissues to heat occurs following x-raying, this method of attacking malignant growths may prove of value. In the present experiments it was demonstrated that active cells such as those from the logarithmic phase are not only more sensitive to x-rays than the less active cells from the stationary phase, but they are also more sensitive to heat. A differential sensitivity therefore exists between active and inactive cells. In malignant growths it may prove possible to selectively affect the actively dividing cells by raying and heat.

<div align="center">SUMMARY</div>

1. *Paramecium caudatum* is sensitized to heat by sublethal dosages of x-rays. Thus if paramecia are irradiated, then exposed to a sublethal dosage of heat

they are killed, but if the same heat exposure precedes the same dosage of radiations, they are not.

2. Sensitivity to both heat and x-rays is much greater in paramecia from the log growth phase than in those from the stationary phase of a culture.

3. Recovery from heat sensitization in animals from the stationary phase of a culture is slow, requiring several days.

4. Division is readily retarded and even temporarily inhibited by sublethal dosage of x-rays. Recovery of the division rate is fairly slow requiring several days.

5. Paramecia can be killed by a dosage of 1,200,000 r (of which about one-half reach the animal) units of x-radiation alone. Smaller dosages are not lethal if the paramecia are transferred to fresh medium immediately upon completion of irradiation.

6. The possibility of utilization of heat sensitization in treatment of malignant growths is discussed.

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PENETRATION OF RADIOACTIVE SODIUM AND CHLORIDE INTO CEREBROSPINAL FLUID AND AQUEOUS HUMOR

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Through the use of Na^{24} as tracer the rate of entrance into the anterior chamber and cerebrospinal fluid of rabbits and dogs after intravenous injection was measured and reported by Visscher and Carr (1). It was shown that Na^{24} passed into the aqueous humor about twice as rapidly as into the cerebrospinal fluid, reaching 75 per cent of equilibrium in 45 minutes. Earlier studies on the rabbit by Kinsey *et al.* (2) indicated an apparent rate of entry from the blood into the anterior chamber equivalent to 4.5 c. mm. per minute of whole aqueous humor. In addition they found that the aqueous humor:plasma concentration ratio for Na^{24} and Cl^{38} reached 50 per cent after approximately 40 minutes following intraperitoneal injection. The rate of entry of P^{32} was found to be less rapid.

Greenberg *et al.* (3) compared the rates of penetration of P^{32}, Na^{24}, K, Br Sr, Rb, and I^{131} into the cerebrospinal fluid after intraperitoneal injection. They used the open drainage method to avoid dilution of the preformed fluid by the newly formed fluid. The cerebrospinal fluid in the present investigation was not disturbed prior to the first cisternal puncture.

The present study follows up the work of Visscher and Carr (1) but is more comprehensive in that it is directed not only to the study of the rates of penetration of radioactive Na and Cl ions across the blood–aqueous humor and blood–cerebrospinal fluid barriers, but also to the study of the rate in which these isotopes diffuse from the blood stream into the extracellular tissue fluid, and of the chloride and sodium space in the dog with the aid of radioactive sodium and chloride.

Apparatus

For the rapid measurement of the radioactivity in the various fluids withdrawn from the animal, a special "immersion" type of Geiger-Müller counter was designed (4). This eliminates the drying process, and the rapidly diminishing radioactivity of Cl^{38}, whose half-life is 37 minutes, could be determined as soon as the fluid was withdrawn from the animal. The wall of this counter is thin enough so that the β-rays can penetrate it easily (thickness, about 0.01 mm.). Several test experiments made with $Na^{24}Cl$ in solution gave a linear relationship between the counting rate and radioactive concentration.

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The power unit consists of two parts: (*a*) the high voltage pack for the Geiger-Müller counter and (*b*) the low voltage pack for the plate and grid-bias voltages. A voltage-stabilizing unit eliminates any fluctuation in line voltages to give a constant Geiger-Müller counter voltage.

A Neher-Harper counting circuit was used because of its high speed. To scale down the counts so that the mechanical counter would not be paralyzed, a scale-of-four unit was added. Thus the mechanical counter recorded only every fourth radioactive particle.

Procedure

Preparation of Na^{24} and Cl^{38}.—The artificially radioactive isotopes, Na^{24} and Cl^{38}, were generated under the influence of accelerated deuterium ions in the Van de Graaf electrostatic generator of the Physics Department of the University of Minnesota (5). The reactions are as follows: —

$$D^2 + Na^{23} \rightarrow H^1 + Na^{24}$$
$$D^2 + Cl^{37} \rightarrow H^1 + Cl^{38}$$
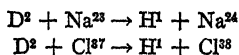
The sodium target was in the form of NaOH, and the chlorine target was $CaCl_2$. After bombardment, the former was neutralized with HCl while the latter was treated with H_2SO_4:

$$CaCl_2^{38} + H_2SO_4 \rightarrow HCl^{38} + Ca_2SO_4.$$
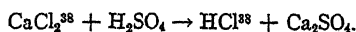
The HCl^{38} was distilled over and neutralized with NaOH:

$$HCl^{38} + NaOH \rightarrow NaCl^{38} + H_2O.$$
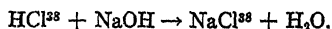
Injection and Drawing of Fluids.—The radioactive salt solution was diluted to isotonic concentration or 0.9 per cent, measured, and injected intravenously into the anesthetized dog. A 5 per cent sodium pentobarbital solution was the anesthetic used. The time of injection was noted, and all the subsequent radioactive measurements that followed were measured from this time. From the measurement of the radioactivity, the concentration of each injected solution was determined.

Blood, cerebrospinal fluid, and aqueous humor were withdrawn at various intervals following the injection, the blood through a vein, the cerebrospinal fluid through a cisterna magna puncture, and the aqueous humor through the cornea. The radioactivity in the cerebrospinal fluid and aqueous humor was measured immediately following their withdrawal from the animal and the radioactivity in the plasma of each blood sample was measured immediately following separation from the corpuscles.

Certain precautions were taken while measuring these fluids. An equal amount of fluid from each sample was used in the radioactive measurements. The positions of the tube containing the fluid and the immersed portion of the Geiger-Müller counter were kept the same for all the measurements. The radioactive decay of each sample was followed through to check the purity of the radioactive elements. The blood samples were carefully handled and centrifuged so that the plasma contained no

hemolyzed corpuscles. Background radioactive counts were recorded at regular intervals.

EXPERIMENTAL RESULTS AND DISCUSSION

A total of eighteen experiments was performed on six dogs of various sizes and weights. In eleven of these experiments Cl^{38} was traced and in seven Na^{24} was traced.

In Table I the results of a typical experiment are shown. It will be noted in Fig. 1 that the initial rapid drop of radioactive ion concentration in plasma is followed by a fairly steady value. The concentrations of these ions in the cerebrospinal fluid and aqueous humor increase slowly to approach that of

TABLE I

The Results of a Typical Experiment

The amount of Na^{24} or Cl^{38} is expressed as the number of radioactive particles passing through the counter per minute corrected to zero time.

Time	Cerebrospinal fluid		Aqueous humor	
	No. of particles	Per cent of plasma level	No. of particles	Per cent of plasma level
min.				
12	312 ± 8	4.5		
27			3460 ± 48	49.4
38	1046 ± 24	15.4		
63	1510 ± 25	22.2		
89			4080 ± 140	60.0
104	3118 ± 108	46.0		
168	5385 ± 163	79.2		

plasma (Figs. 2 and 3). The solid lines in these figures are drawn from the following theoretical exponential equation:[1]

$$C_a = C_p (1 - e^{-kt})$$

where C_a = concentration of the radioactive ion in cerebrospinal fluid or aqueous humor at time t, C_p = concentration of the same ion in plasma at t, t =

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[1] The derivation for the equation is as follows:—

Consider the anterior chamber (or cerebrospinal canal) as having two openings, one for inflow from the blood stream by either ultrafiltration, secretion, or both, the other for outflow into the blood by the same mechanisms. The first may be expressed by the equation:

$$\left(\frac{dQ}{dt}\right)_1 = k_1(H_2 - F)C_p \tag{1}$$

the second by:

$$\left(\frac{dQ}{dt}\right)_2 = k_2(H_2 - F)C_a \tag{2}$$

time interval after injection of the radioactive salt, and k = net coefficient of transfer from plasma to cerebrospinal fluid or aqueous humor. The constant

where Q = amount of Na^{24} or Cl^{38} present in the aqueous humor (or cerebrospinal fluid);

C_p = concentration of these ions in the plasma;

C_a = concentration of these ions in the aqueous humor (or cerebrospinal fluid);

k_1, k_2 = coefficients of transfer into and out of the aqueous humor (or cerebrospinal fluid) by flow (ultrafiltration, secretion, or both);

H = hydrostatic pressure in blood stream;

F = colloid osmotic pressure in blood stream;

t = time.

Diffusion processes occurring simultaneously at these openings may be expressed thus:

$$\left(\frac{dQ}{dt}\right)_3 + \left(\frac{dQ}{dt}\right)_4 = (k_3 + k_4)(C_p - C_a)$$

or

$$\left(\frac{dQ}{dt}\right)_{3,4} = k_{3,4}(C_p - C_a) \tag{3}$$

where subscripts 3 and 4 represent the *into* and *out of* processes respectively. The sum of (1), (2), and (3) is:

$$\left(\frac{dQ}{dt}\right) = k_1(H_1 - F)C_p + k_2(H_2 - F)C_a + k_{3,4}(C_p - C_a) \tag{4}$$

Since at equilibrium the outflow is equal to the inflow, then

$$k_1(H_1 - F) = -k_2(H - F). \tag{5}$$

Therefore,

$$\begin{aligned}\left(\frac{dQ}{dt}\right) &= \left(\frac{dQ}{dt}\right)_{1,2} + \left(\frac{dQ}{dt}\right)_{3,4} \\ &= k_1(C_p - C_a)(H_1 - F) + k_{3,4}(C_p - C_a) \\ &= K(C_p - C_a) \end{aligned} \tag{6}$$

where $K = k_1(H_1 - F) + k_{3,4}$.

VC_a is substituted for Q in equation (6), where V is the volume of the anterior chamber (or cerebrospinal canal), and we have

$$\frac{VdC_a}{dt} = K(C_p - C_a),$$

which, when terms are transposed and the equation integrated, becomes

$$C_p - C_a = Be^{(-Kt/V)}. \tag{7}$$

At $t = 0$, $C_a = \dfrac{Q}{V} = 0$, and $C_p = B$, a constant. Equation (7) becomes

$$\begin{aligned}C_a &= C_p(1 - e^{(-Kt/V)}) \\ &= C_p(1 - e^{-kt})\end{aligned}$$

where $K/V = k$, the net coefficient of transfer. At $t = \infty$, $e^{-kt} = 0$, and $C_a/C_p = 1$.
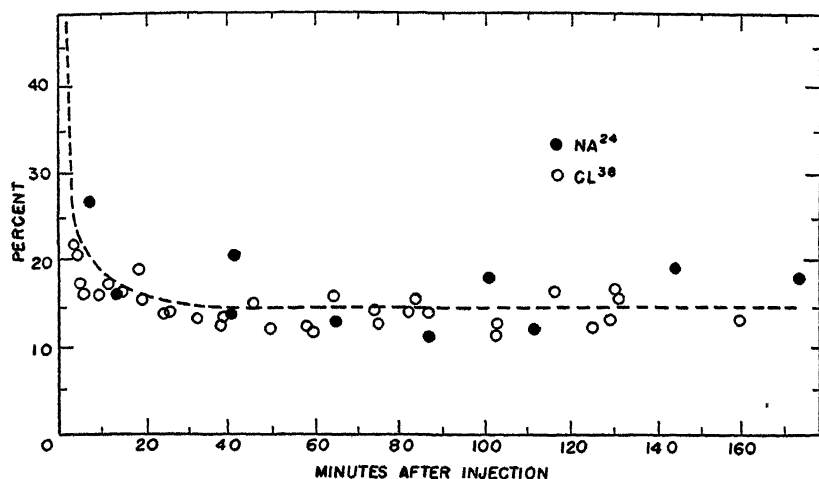
FIG. 1. Per cent of radioactivity remaining in plasma at various times after injection.
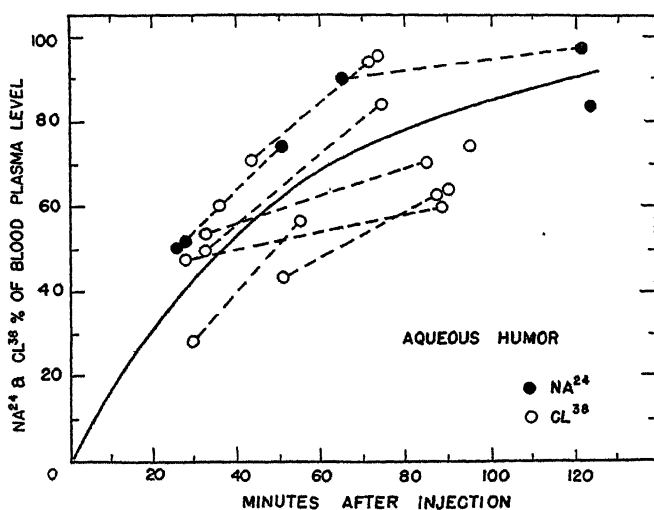
FIG. 2. Radioactive sodium and chloride concentration in the aqueous humor of dogs in per cent of the simultaneous plasma level as a function of time after Na^{24} and Cl^{38} injection. The broken lines connect the points representing experimental values obtained from the aqueous humor from the two eyes in a single experiment. The solid line is the theoretical curve for the equation $C_a = C_p (1 - e^{-kt})$ when the mean value of k is applied.

k is calculated from the half-value interval $t_{0.5}$ or the time required for the radioactive ion concentration in the various fluids to reach 50 per cent of that
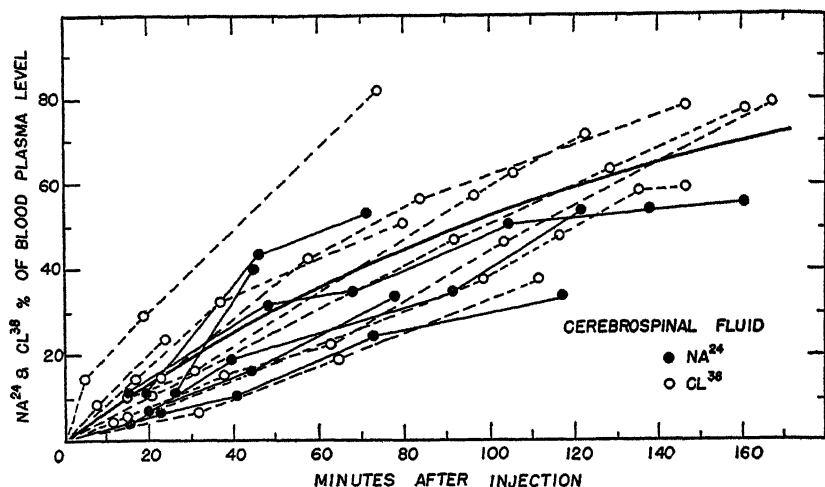
FIG. 3. Radioactive sodium and chloride concentration in the cerebrospinal fluid of dogs in per cent of the simultaneous plasma level as a function of time after injection. The broken and the light solid lines connect values determined in a single experiment and the heavy solid line is the theoretical curve for the equation $C_a = C_p (1 - e^{-kt})$ when the mean value of k is applied.

TABLE II a

The Half-Value Interval $t_{0.5}$ and the Net Coefficient of Transfer k for Na^{24}

Radioactive ion	Experiment No.	Cerebrospinal fluid		Aqueous humor		
		$t_{0.5}$*	k	Eye	$t_{0.5}$*	k
		min.			min.	
Na^{24}	1	45	0.0174	Right	28 ($t_{0.521}$)	0.0262
	1			Left	51 ($t_{0.752}$)	0.0273
	4	118	0.0059			
	8	63	0.0110			
	9	118 ($t_{0.334}$)	0.0035	Left	125 ($t_{0.810}$)	0.0133
	11	78 ($t_{0.239}$)	0.0053	Right	26 ($t_{0.610}$)	0.0188
	14	104	0.0067	Left	65 ($t_{0.902}$)	0.0357
	14			Right	122 ($t_{0.978}$)	0.0311
		Mean $k = 0.0073 \pm$ 0.0005		Mean $k = 0.0254 \pm 0.0008$		
		Mean $t_{0.5} = 95 \pm 6$ min.‡		Mean $t_{0.5} = 27.3 \pm 0.9$ min.‡		

* The values are for $t_{0.5}$ (the half-value interval) unless otherwise specified. In the latter cases the $t_{0.5}$ was not obtainable.

‡ The mean $t_{0.5}$ was calculated from the mean k.

in plasma. Table II gives the $t_{0.5}$ and k for each experiment. The constant k for aqueous humor was determined not for each experiment but for each eye

from which aqueous humor was drawn. This was necessary because only a single paracentesis from each eye was possible in any one experiment, and the $t_{0.5}$ could not be ascertained since the drawing of a complete curve was not possible. The subvalue for each time t is the ratio of radioactive ion concentration in aqueous humor to the simultaneous concentration in plasma. The average of sixteen values gives a mean net coefficient of transfer k for chloride from

TABLE II *b*

The Half-Value Interval $t_{0.5}$ and the Net Coefficient of Transfer k for Cl^{38}

Radioactive ion	Experiment No.	Cerebrospinal fluid		Aqueous humor		
		$t_{0.5}$*	k	Eye	$t_{0.5}$*	k
		min.			*min.*	
Cl^{38}	3	24	0.0114	Left	29 ($t_{0.280}$)	0.0110
	3			Right	56 ($t_{0.580}$)	0.0153
	5	41	0.0169	Left	96 ($t_{0.743}$)	0.0167
	6	36 ($t_{0.189}$)	0.0059	Left	43 ($t_{0.70}$)	0.0279
	6			Right	74 ($t_{0.89}$)	0.0298
	7	100	0.0069	Right	72 ($t_{0.91}$)	0.0334
	10	79	0.0088	Left	90 ($t_{0.824}$)	0.0109
	12	62	0.0024	Left	33 ($t_{0.539}$)	0.0234
	12			Right	85 ($t_{0.704}$)	0.0144
	13	112 ($t_{0.135}$)	0.0042	Right	51 ($t_{0.430}$)	0.0110
	13			Left	88 ($t_{0.625}$)	0.0111
	15	112	0.0062	Right	27 ($t_{0.494}$)	0.0252
	15			Left	89 ($t_{0.600}$)	0.0103
	16	121	0.0057	Left	36 ($t_{0.600}$)	0.0254
	17	85	0.0081			
	18	73	0.0095	Right	33 ($t_{0.500}$)	0.0210
	18			Left	75 ($t_{0.842}$)	0.0245

		Mean k = 0.0077 ± 0.0005 Mean $t_{0.5}$ = 90 ± 6 min.‡	Mean k = 0.0194 ± 0.0008 Mean $t_{0.5}$ = 34.3 ± 0.9 min.‡

* The values are for $t_{0.5}$ (the half-value interval) unless otherwise specified. In the latter cases the $t_{0.5}$ was not obtainable.

‡ The mean $t_{0.5}$ was calculated from the mean k.

plasma to aqueous humor equal to 0.0194 ± 0.0008. In practical units this is equivalent to 1.94 ± 0.08 per cent per minute increase in concentration of radiochloride in the aqueous humor relative to that in the plasma. A more convenient form of expressing rate is the half-value interval which for this particular instance is 34.3 ± 0.9 minutes. Since the average volume of the anterior chamber is about 270 c. mm. the rate of entry is then calculated to be approximately 4 c. mm. per minute. Corresponding values for Na^{24} are 2.54 ± 0.08 per cent per minute for k, 27.3 ± 0.9 minutes half-value interval, and 5 c. mm. per minute for the rate of entry. Kinsey *et al.* (2) reported an apparent

rate of entry of sodium and chloride from the blood into the anterior chamber following intraperitoneal injection equivalent to 4.5 c. mm. per minute of whole aqueous humor, whereas heavy water (D_2O) in the blood stream enters the anterior chamber at a rate equal to approximately 50 c. mm. per minute (6). No value for the rate of accumulation of radiophosphorus was given, but it was stated that the rate is less rapid. In another paper (7) these same authors reported rapid rates of accumulation and high aqueous:blood ratios of SCN and bromides, and slower rates and lower ratios for PO_4, urea, lithium, and levulose.

Table II also shows the mean k and $t_{0.5}$ values for the cerebrospinal fluid obtained through the cisterna magna. The mean net coefficients for Cl^{38} and Na^{24} are 0.77 ± 0.05 per cent per minute and 0.73 ± 0.05 per cent per minute, respectively. The half-value intervals are calculated to be 90 ± 6 minutes and 95 ± 6 minutes, respectively. These findings agree with those of Visscher and Carr (1). Greenberg et al. (3) using the open drainage method[2] to diminish dilution of the newly formed spinal fluid with the preformed fluid found a rate of flow averaging 0.2 ml. per hour per kilo body weight of dogs. The relative CSF:plasma Na^{24} ratio reached 60 per cent at 1 hour, 80 per cent at 2 hours, and 110 per cent at 20 hours. No chloride values were reported. Radiophosphorus P^{32} in cerebrospinal fluid reached its maximum concentration of 23 per cent at 2 to 2.5 hours. Radiopotassium concentration reached its maximum of 50 per cent in 30 minutes, then increasing at a slower rate to approach 62 per cent at 18 hours. These same authors summarized the CSF:plasma concentration ratios of labelled ions in the following order:

$$K > Na > Br > Rb > Sr > PO_4 > I$$

The percentage of radioactive ions remaining in the plasma at various times after injection of the salt is shown in Fig. 1. The concentration of Na^{24} and Cl^{38} in the animal immediately following this injection was calculated from the total amount of radioactive salt injected and the assumed plasma volume which is on the average 4.9 per cent of the animal's body weight. The following equation was used:

$$\text{Radioactive counts per unit of volume plasma} = \frac{\text{Total counts injected}}{4.9 \times \text{body weight}}$$

This value represented the initial 100 per cent. The distribution of the values that follow is plotted in Fig. 1. They show that Na^{24} and Cl^{38} rapidly diffuse out of the blood stream into the various tissues. Thus, 4 minutes after the injection the concentration of these ions remaining in the plasma dropped to about 22 per cent of its initial concentration, but during the next 15 minutes the rate of loss was less marked. A steady state was established at about 15 per cent

[2] This open drainage method is not comparable to the method used in this investigation.

after 25 minutes. This type of curve has been reported by Hevesy, Holst, and Krogh for P[82] (8), by Manery and Bale (9), Griffiths and Maegraith (10), Merrill, Gellhorn, and Flexner (12), and Greenberg et al. (3) for Na[24], and Manery and Haege (11) for Cl[38]. Merrill et al. gave for guinea pigs a value of 13 per cent total Na of extravascular fluid being exchanged. Manery and Bale calculated the extracellular fluid as measured for Na[24] to be 29 per cent of the body weight in rats and 25 per cent in rabbits. Greenberg et al. found that Na[24] attained equilibrium with about 24 per cent of body water of dogs. Potassium thiocyanate on the other hand diffuses into the extracellular fluid at a slower rate than Na[24] or Cl[38], requiring from 47 to 67 minutes to reach a stabilized level in the plasma (13). The fact that a steady state was established within such a short time for the electrolytes indicates a rapid exchange rate across the capillary walls.

From these data the per cent of volume occupied by solutions containing Na[24] and Cl[38] was calculated by means of the following equation:

$$\text{Na space} = \frac{\text{Total counts injected} \times 100}{\text{Counts per ml. at } t \times \text{weight of animal}}$$

The results are only apparent values for losses in excretion, secretion, and unequal distributions go on continuously. The distribution of values after equilibrium has been reached was calculated to be 33 ± 5 per cent. This agrees within the limits of error with the result reported by Griffith and Maegraith who used the same method of calculation in their experiments on rabbits.

SUMMARY

1. Experiments were performed on six dogs to determine the rate of penetration of Cl[38] and Na[24] across the blood–aqueous humor and blood–cerebrospinal fluid barriers after intravenous injection of the radioactive ions. The radioactivity measurements were made with an immersion type of Geiger-Müller counter.

2. The concentrations of the labelled ions in the anterior chamber and the cisterna magna increase slowly to approach that of plasma. The rate of penetration k is calculated from a simple exponential equation with the half-value interval $t_{0.5}$ or the time required for the labelled-ion concentration in the fluid to reach 50 per cent of that of plasma. The average $t_{0.5}$ for Cl[38] and Na[24] in aqueous humor are 34.3 ± 9 and 27.3 ± 9 minutes, respectively, while those for cerebrospinal fluid are 90 ± 6 and 95 ± 6 minutes, respectively.

3. A study of the radioactivity in plasma was made to determine the per cent remaining after a steady state was reached. By means of this determination the sodium and chloride space was calculated to be 33 ± 5 per cent.

his assistants for the radioactive isotopes, to Mr. J. F. Marvin for his assistance in the construction of the electrical counting unit, and to Dr. W. Armstrong, Dr. N. Lifson, Dr. C. Carr, and Dr. W. Kubicek for their advice and assistance.

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THE QUANTIC AND STATISTICAL BASES OF VISUAL EXCITATION

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At the present time we are still very far from having an entirely satisfactory theory of the mechanism of visual excitation.

In order to study this mechanism objectively we must take threshold excitation as the starting point, because it is there that the conditions are the simplest. Indeed, several authors have been led to formulate empirical laws, but none of these laws could provide a valid interpretation of the facts over the whole range of brightness. On the other hand, with none of the different theories is it possible to compute quantitatively the course of the phenomena investigated, unless one makes use of one or several parameters arbitrarily chosen so as to make the theory fit the experiments.

Contrary to the theories hitherto proposed, it seems obvious that in vision several mechanisms are involved and that they come into play one after another as brightness increases. *In the present paper we shall put forward a theory which attempts to give a quantitative explanation of the liminal phenomena of vision without involving any arbitrary parameter.*

This theory provides a basis for the further analysis of many other problems concerning supraliminal levels, especially those of brightness discrimination and visual acuity.

Absolute Threshold

We are now well aware that the photochemical theory of vision can at most explain the facts at the average levels of brightness only. The outstanding work of Hecht, Shlaer, and Pirenne seems to deny the possibility of explaining the mechanism of visual excitation by the mass action law. Indeed, according to these authors, 5 to 7 quanta, absorbed by receptor cells situated in a peripheral retinal area, subtending a visual angle of 10', are the upper limit of the number of quanta necessary for producing a visual sensation, 60 out of 100 times. The number of rods contained in the above area (region IV, near periphery) is about 500, and the probability that 2 or more quanta are absorbed in the same rod is so slight that we may conclude that when the excitation is liminal, only one molecule of retinal purple is decomposed in each rod absorbing light. Now the photochemical theory claims that the level of sensation is conditioned by the degree of concentration of a certain substance produced by the decomposition of the visual purple; since the existence of absolute thresh-

old is a fact that cannot be denied, this threshold must correspond to a certain degree of concentration of that substance (liminal concentration). But the decomposition of a single molecule of visual purple in each rod absorbing light cannot provide more than a single molecule of efficient substance, perhaps even no more than a single atom or a single electron, for we know nothing about this entirely hypothetical substance. The only thing we are sure of is that, if it does exist, its molecular weight must be very slight in comparison with that of visual purple, for the molecular weights of the three purples, the visual, the yellow, and the white, have been found to be the same. It is therefore obvious that the mass action law—which is essentially a statistical law—cannot come into play at the threshold level. This law—and therefore the photochemical theory—cannot then account for the facts unless brightness is such that the average number of decomposed purple molecules in each rod is a non-negligible part of the total number of visual purple molecules contained in one single rod.

But this is the case only when the brightness reaches about 1 millilambert and the absolute threshold is about 10,000 times lower, even if one allows for pupillary aperture variation.

Afterwards Van der Velden found that 2 quanta may produce a liminal sensation when they are absorbed in a small peripheral retinal area and the time interval between them does not outlast a certain constant. *This constant is furnished by experiment.* Van der Velden uses a statistical method already employed by Hecht, Shlaer, and Pirenne. It consists in computing the number of positive responses in relation to the logarithm of stimulus brightness. The curve thus obtained must be a Poisson exponential expansion curve, characteristic of the number n of independent events, that is of quanta necessary to induce excitation. Indeed, as n is a small number, the average number N of quanta absorbed during a flash will not always be equal to n. On account of the chance distribution of the quanta contained in a small flux, q quanta (q: $0, \ldots, n-1, n, n+1, \ldots$) will be absorbed and Poisson's law makes it possible to compute the probability $P_{N, q}$ that the number of quanta absorbed during a single test will be equal to q:

$$P_{N,q} = N^q / e^N q!$$

e being the basis of Naperian logarithms. When the retina absorbs more than n quanta, of course we also experience a visual sensation. We must therefore compute the probability for n *or more* quanta to be absorbed, among those carried by a flash which on the average provides N quanta to the retina. For each value of n, we thus obtain a definite Poisson curve, steeper and steeper as the parameter n increases. Fig. 1 shows a "family" of these curves in which the abscissae are fractions and multiples of N_l, N_l, being the average number of quanta absorbed by the retina when the flash intensity is liminal,

and the ordinates percentages of the probability for q to be superior or at least equal to n.

In order to compute n, we must use stimulus intensities which will vary from a fraction to a multiple (*e.g.* twice) of the liminal intensity. Then, for each brightness b given, we write down the proportion of positive responses, the corresponding abscissa being b/b_l when b_l is the liminal brightness.

The curve thus obtained must coincide with one of the graphs of Fig. 1. This method led Van der Velden, experimenting on two observers, to state that $n = 2$. Van der Velden uses as abscissae the logarithm of b; Hecht, Shlaer,
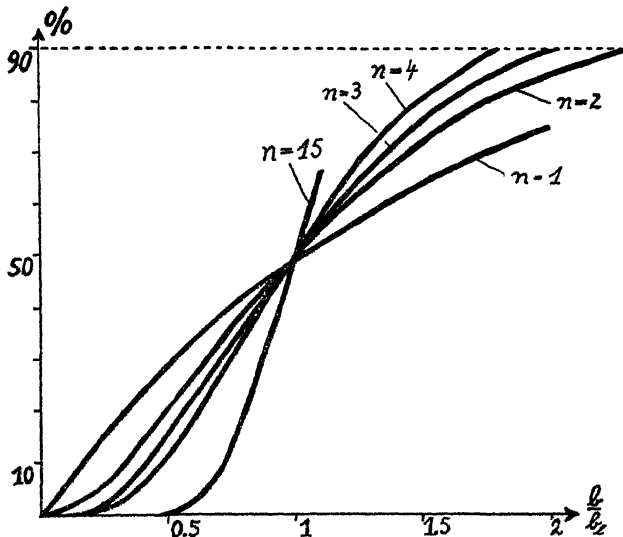
FIG. 1. Poisson's law. The proportion of positive responses to weak stimuli in relation to stimulus brightness and number n of quanta necessary to induce a sensation. Threshold brightness is chosen as abscissa unit.

and Pirenne use the logarithm of the average number of quanta carried by a single flash. In order to find the number of quanta absorbed by the rods, Hecht, Shlaer, and Pirenne measure the mean energy of the liminal flash, compute losses by reflection and absorption between cornea and retina, and suppose that the concentration of visual purple in the rods is *at most* 20 per cent; thus they compute 5 to 14 quanta. But their graphs show that an 8 per cent concentration is the most probable one. If we choose the 8 per cent hypothesis, we compute 2 to 6 quanta instead of 5 to 14 quanta. The fact that other runs, interpreted according to Poisson's law, furnished to the authors a number n varying from 5 to 7 does not seem contradictory. Indeed, if excitation does take place, a sensation does not follow necessarily; we observed

that, depending upon his physical and psychological condition, one of our subjects saw the flash one day when his retina absorbed 4 quanta, another day when it absorbed 10 quanta, experiments always being preceded by a 50 minute adaptation.   Training also plays an important part, and in order to find n we must not use average values but select and train the observers beforehand.   Fig. 2 shows quite a homogeneous run which furnished the value of $n = 5$.   The best performances of the best observers will give a number n which will be the absolute threshold.   Therefore it seems justifiable to accept 2 quanta as sufficient to excite, that is to induce a propagated action potential.   But this excitation will not necessarily produce a sensation.
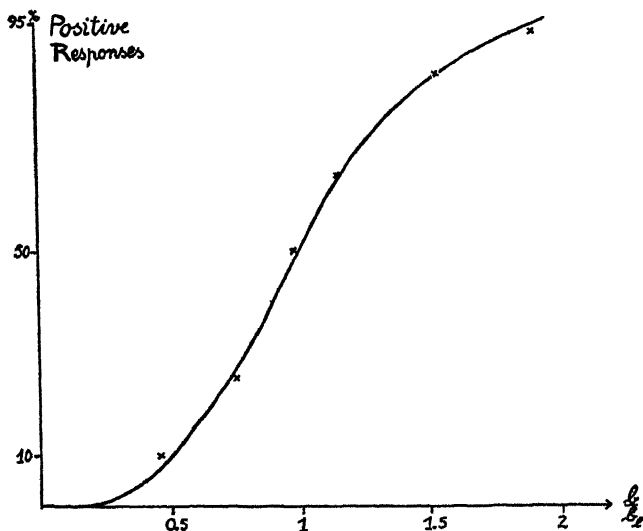
FIG. 2. An example of threshold measurement.   The solid line is the Poisson curve for $n = 5$.

The above method is justified when the number of quanta absorbed by the retina is so small that error of measurement cannot mask the shape of the curve (see Fig. 1).   On the other hand, it does not assume the knowledge of the mean number of quanta contained in one flash, nor the mean number N of quanta absorbed by the retina.   If we take Van der Velden's result as the basis of our calculation, we shall not be contradicted by any fact observed in sensory physiology or neural physiology.   Without formulating any hypothesis concerning the inner mechanism of visual excitation, we are now able to aim at a *numerical* solution of the problems of liminal visual excitation.

The Conditions of Liminal Excitation

According to several authors, among whom we shall quote only Blondel and Rey, Piéron, and Graham and Margaria, one always notes a time τ which de-

pends on the size of the test area and on the position of that area on the retina, and is such that $bt =$ constant when $t \leqq \tau$. This applies to foveal as well as to peripheral vision, and to large retinal areas as well as to very small ones. If we take into account this fact on one hand and Van der Velden's result on the other, we are led to express the conditions of liminal excitation as follows:—

Liminal excitation is caused by the absorption of 2 quanta of light, happening, in a time interval $t \leqq \tau$, inside a retinal area to which we shall give the name of *quasi-independent unit*. This unit is such that in each region of the retina it is the largest area possible within which Ricco's law ($bS =$ constant) is valid; τ is a time constant which is to be found experimentally.

Thus we see that the quantic and statistical theory of visual excitation which we here put forward does not assume any "ad hoc" mechanism nor any arbitrary parameter.

Liminal Brightness in Relation to the Size of the Retinal Area Stimulated—Ricco's, Piper's, and Piéron's Laws

Ricco's law is an expression of the fact that, given certain space-time conditions, we observe what is usually called "total summation." According to this law, liminal intensity varies inversely to the stimulated areas; that is to say, when we use quantic terminology, the number of quanta likely to induce liminal excitation is a constant whatever the size of the retinal area stimulated. Nowadays we know that Ricco's law is not valid for all the regions of the retina nor for areas of every size. The most outstanding exceptions are the foveal region and any peripheral area above a certain size which varies according to the retinal region involved (Piéron; Graham and Margaria). For instance, in region IV (near periphery) which spreads between 12°30' and 20° from the foveal center, Ricco's law is strictly valid for circular test areas, the visual angle of which varies from about 2' to 1° (see Graham and Margaria and this paper, p. 281). This has been tested with white and blue light.

Let us therefore suppose, for the time being, that any retinal region is made up of quasi-independent units (according to our definition of this term, see above). In this case, liminal excitation can only take place when 2 quanta are absorbed, during a time interval t sufficiently small, by two rods belonging to the same unit; that unit contains, in this retinal region, about 18,000 rods (Østerberg).

When the test area involves several of these units, by which law, then, should Ricco's law be replaced?

Let us call k the number of these stimulated units, and compute the probability that 2 quanta (or more) among q will be absorbed by the same unit, q being the number of quanta absorbed in the test area. This probability is equal to 1 minus the probability that the opposite event does happen; that is to say, that each of the q quanta is absorbed by a separate unit.

The function $P_{k,a}$ has this particular characteristic, that when k is not too small, its value remains practically constant when one multiplies k by a and q by $\sqrt{a}$, where a is any positive number. It follows that the liminal energy increases as the square root of the test area when the latter contains several of those units.[2] This is Piper's law which, empirical at first and verified only in certain conditions of area, region, and time, becomes now a basic law, valid everywhere that we deal with a homogeneous population of units, inside which Ricco's law is valid.

Indeed, Graham and Margaria have observed that Piper's law holds good for the retinal region investigated by them, when the visual angle of the test area varies from 1 to 3° in diameter. Since they have not used larger areas, we still have to prove that the same law applies also when the stimulated areas have larger diameters, but not larger than the limits of the investigated retinal region.

Let us now examine the course of liminal energy as influenced by the size of the area, inside the outer fovea, the diameter of which is 1.20′ (Polyak). If the cones were a homogeneous population, Piper's law would be valid over the whole range of the outer fovea. But this is not so. In the very center of the fovea, the diameter of the cones is minimum and it increases towards the foveal periphery.

When we measure the foveal threshold for red light, for areas of increasing size, we must expect that Piper's law will be affected according to the density of the cones. On account of the fact that the cone diameter increases from the center towards the edge of the fovea, the threshold intensity must decrease more slowly than the square root of the ratio of the stimulated areas increases.

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[1] The first quantum may be chosen between k units, the second between $k-1$, ... the qth between $k-q$." The product $k(k-1)(k-2)\ldots(k-q)$, which is the number of favorable combinations, may be written

$$\frac{k!}{(k-q)!}$$

to Østerberg is 1,050, 3,050, and 9,100. Polyak's data allow us to compute the number of cones contained in circles respectively 50, 125, and 200 μ in radius. These numbers are the following: 2,250, 9,350, and 18,600.

In order to compute the coefficient of spatial summation, let us apply Piper's law. If n_{a_1} is the number of cones contained in a circle, the radius of which we shall call a_1, and if n_{a_2} is the number of cones contained in a second circle, the radius of which we shall call a_2, $a_2 > a_1$, according to Piper's law we shall have:

$$\frac{b_{a_1}}{b_{a_2}} = \sqrt{\frac{n_{a_2}}{n_{a_1}}} = \left(\frac{n_{a_2}}{n_{a_1}}\right)^{0.5}$$

When replacing the ratio $\dfrac{n_{a_2}}{n_{a_1}}$ by $\dfrac{S_{a_2}}{S_{a_1}}$, where S_{a_1} and S_{a_2} are respectively the areas of the two circles, we may compute easily:

$$\frac{b_{a_2}}{b_{a_2}} = \left(\frac{S_{a_2}}{S_{a_1}}\right)^{0}$$

Now Piéron, for areas from 6' to 2° in diameter, finds 0.30 the summation coefficient; Elsberg and Spotnitz, for areas from 8' to 36' in diameter, find a coefficient varying between 0.33 and 0.30. Let us note that Piéron, as well as Elsberg and Spotnitz, has experimented with stimuli lasting several seconds, which affects the results because of fixation-micronystagmus effect. Now, when the tested areas are small, this effect is relatively greater than it is for bigger areas, and it seems possible to ascribe to it the numerical gap between theory and experience. We intend to take up again in the near future the same investigations, but with short flashes as stimuli, in order to check whether this effect is actually the only cause of the observed gap.

Here we must raise the question: what is, inside the fovea, the nature of the quasi-independent unit? Foveal histology teaches us that it is more than probable that each foveal cone is linked to its own ganglion cell by the medium of its individual bipolar cell. We are, then, justified in surmising that the quasi-independent foveal unit is made up of one cone only or, at least, of a small

number of cones forming a functional color vision unit. The comparison of the peripheral and foveal thresholds, interpreted by means of probability calculations, will provide us with a definite answer, but it would take too long to discuss this important question here, and we shall deal with it in a future paper.

At any rate we are justified in stating that Elsberg and Spotnitz's observations, relative to very small foveal areas, cannot provide a satisfactory answer to this question. Indeed, their experimental conditions are not guaranteed against the intervention of two artefacts; *viz.*, micronystagmus and fixation point intervention. It will indeed be useful to take up their observations again, however difficult it is, for they are liable to provide very valuable information.
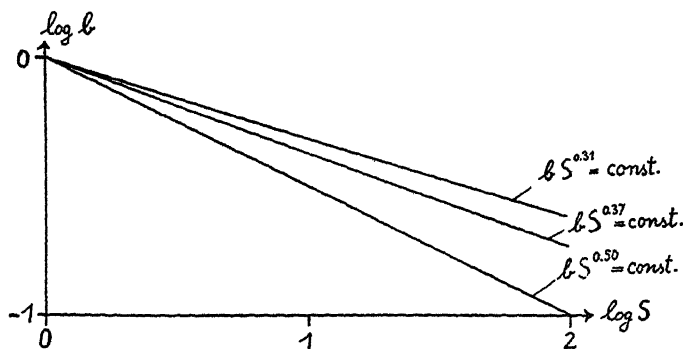
FIG. 3. Foveal threshold brightness b in relation to stimulated area S. Piper's law: $bS^{0.50}$ = constant; relation computed theoretically: $bS^{0.37}$ = constant; experiments (Piéron, Elsberg and Spotnitz): $bS^{0.31}$ = constant.

Threshold Brightness in Relation to Stimulation Time and Size of Test Area

If b is the liminal brightness that corresponds to a stimulation time t, what must be the course of the function $bt = f(t)$?

When made on a well circumscribed retinal region, experiments (Piéron, Graham and Margaria) always yield curves of the same type; bt remains constant up to a certain limit, then rises quite abruptly and continues to rise, but more and more slowly. From the point where they cease to be horizontal straight lines (Bunsen-Roscoe's law) these curves are similar to parabolas (Piéron).

Though it is often asserted that b becomes constant as soon as t reaches a certain value called "summation time" (*temps utile* in French), we have not been able to find any trace of experiments yielding the above result, the only exception being those of Blondel and Rey who operated with mixed (foveal and parafoveal) vision. But these experimental conditions are too complex to yield results liable to be taken into account in the discussion of the present question. Why then do so many authors assert that b becomes constant when t reaches a certain value which they agree to place at about 3 seconds?

The answer seems to be as follows: First, as eye movements increase with fixation time, experimental thresholds will yield too high values when t reaches values of several seconds. And secondly, as bt increases with t, these authors conclude that we have before a temporal summation phenomenon, the "memory" of the responsible neuron decreasing as t increases, and so they are led to state that there is a limit beyond which the neuron needs a constant input of new energy, that input being just sufficient to compensate the loss of energy caused by the weakness of the neuron's "memory."

It is surprising that the parabolic shape of the curves $bt = f(t)$ has not long since incited physiologists to reconsider the above interpretation; the more so since this type of curve is found also in animals such as Mya, which lacks eyes, and in nerve response to electrical stimulation (Lapicque). Recently, Segal
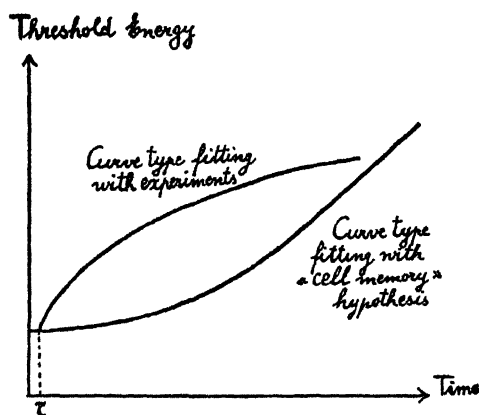
FIG. 4

proved that cell memory, being a passive property, cannot explain this type of curve. Indeed if the classical interpretation were true, such a summation should yield a curve of experimental type in which the energy loss involved by limited cell memory, unnoticeable at first, would become more and more important until, at the end of the "summation time," the curve would become a straight line. Now this classical interpretation applies only to concave curves, but experiments have always yielded convex curves (Fig. 4).

We shall eliminate this contradiction if we give up the hypothesis of temporal summation and analyze the phenomenon from the quantic and statistical point of view.

Threshold Brightness in Relation to Size of Tested Areas

Whatever the inner mechanism of liminal excitation, the quantic structure of light and the random distribution of quanta in weak flashes involve necessarily a certain relationship between threshold brightness b on the one hand

and size S of test area and stimulation time t on the other. Therefore, if the experiments yield numerical results that are the same as those predicted by the quantic and statistical theory, there should be no doubt whatever that the peripheral mechanism of liminal excitation is the same as that involved in this theory.

Table I shows corresponding values of bt and t, relative to test areas subtending visual angles of respectively 2°, 16°, 1°, and 3° (Graham and Margaria).

TABLE I

Relative Threshold Brightness Values in Relation to Stimulation Time and Size of Stimulated Retinal Area (Graham and Margaria)

Stimulation time				
millisec.				
0.31	—	9.34	0.565	0.178
0.63	—	9.55	0.589	0.178
1	195	—	—	—
1.25	—	9.34	0.603	0.187
2	191			
2.5	—	9.55	0.589	0.224
4	191			
5	—	9.78	0.646	0.219
8	195			
10	—	9.35	0.677	0.258
16	200			
20	—	9.78	0.742	0.282
32	200			
40	—	10.5	1.13	0.399
64	191			
80	—	12.9	1.52	0.502
128	252			
160	—	17.0	1.82	0.708
256	390			
320	—	25.2	2.89	0.978
500	742			
640	—	47.9	4.27	1.48

Let us now consider the fourth line of this table. We notice that here bt is still constant for each of the four columns. Piper's law applies quite well between 1° and 3°, but Ricco's law seems to apply only between 16° and 1°, and between 2° and 16° neither applies. This is why we thought it necessary to continue part of Graham and Margaria's investigations.

· Our subjects were M. R., a young man of 17, and F. A., a young girl of 23. Both had normal vision. Each series of runs was preceded by 50 minutes' adaptation in complete darkness. The physical arrangement may be seen in Fig. 5. The light source is a tungsten filament lamp run on a constant current obtained from storage

cells. It sends part of its flux on a dry disc photocell which is connected with a micro-ammeter; thus continuous intensity control is secured. Another part of the luminous flux enters a glass bar, 11 mm. in diameter, the two bases of which are rough. When it comes out of this bar, the luminous flux goes through a slit, the opening of which may be varied from 0 to 10 mm. by means of a micrometrical screw. We used only openings between 1 and 5 mm., for the calibration of the apparatus, operated by means of a dry disc photocell and a galvanometer showed that outside these limits the brightness of the observed field is no longer proportional to the opening of the screw-driven slit. The diffuse light pencil thus produced goes through a second slit per-
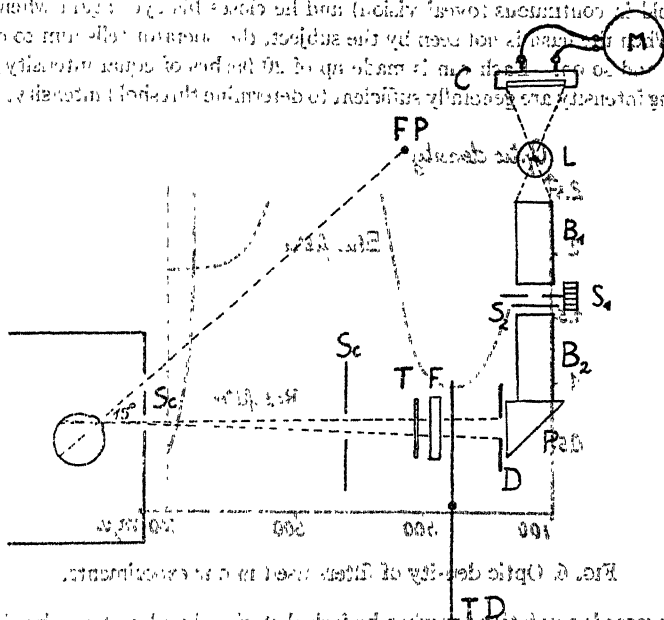
FIG. 5. Apparatus. B_1, B_2, glass bars; C, dry photocell; D, diagram; E, colored filter; FP, fixation point; L, light source; M, microammeter; P, prism; S_1, S_2, slits. Sc, screens; T, neutral glass filter (Tscherning type); TD, turning disc.

pendicular to the first, the opening of which may be 1, 2, or 5 mm. Then the light pencil enters a second glass bar, identical with the first one, at the end of which a total reflecting prism projects the diffuse light on the test field. The area of the latter may be varied by means of diaphragms 2, 3, or 5 mm. in diameter.

The test field is masked to the observer by an ebonite disc which turns round at a constant speed so that the field becomes visible every 9 seconds, during a time which depends on the opening of an adjustable sector of the disc. According to the length of the stimulation time thus determined, the shape of stimulus varies. Indeed, it is sinusoidal for very short stimuli and its plateau becomes longer and longer as the stimulation time increases. If the threshold is determined by nothing but the num-ber of quanta absorbed by the retinal test area during the stimulation time t, as it is

involved by the quantic and statistical theory of excitation, it must be independent of the shape of the stimulus. Experience showed that this is indeed the case. Between the test field and the subject's eye is placed a blue filter which has its transmission maximum at 479 mμ; in red light its transmission is poor (Fig. 6). If necessary, a neutral glass filter (Tscherning type) is used to decrease light intensity.

The subject's chin rests on a support and a screen protects his left eye from any stimulation. When beginning a run, the subject is told to close his eyes and 2 or 3 seconds before the occurrence of the flash he is told to open them. Then he fixates a small red point, the brightness of which is the faintest possible (about 2 to 3 times the threshold in continuous foveal vision) and he closes his eyes again when he has "seen." When the flash is not seen by the subject, the operator tells him to close his eyes again, and so on. Each run is made up of 20 flashes of equal intensity; 6 runs of decreasing intensity are generally sufficient to determine threshold intensity. When
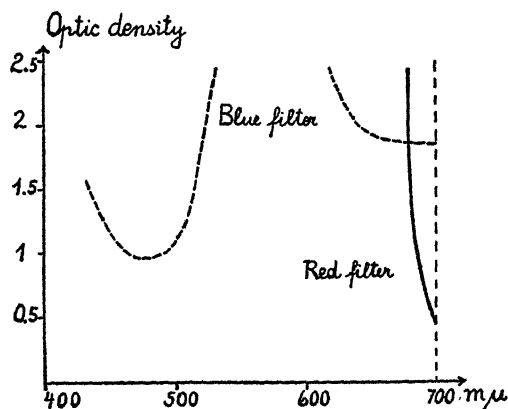
FIG. 6. Optic density of filters used in our experiments.

an observer records any fatigue or when he feels disturbed by phosphenes he closes his eyes for a while, and the run is resumed some minutes later.

Thus we reduce undue fatigue as much as possible, fixation not needing to be continuous. On the other hand, this method eliminates another source of error, scarcely investigated; viz., rapid adaptation to liminal or very faint stimuli. Indeed, when, in peripheral vision, a just perceptible continuous stimulus is given, about 3 seconds later this stimulus is no longer "seen;" that is to say, it has become subliminal. But after closing his eyes for a while, the subject "sees" it again, and so on.

When we began our investigations, we asked the subject to wear before one eye an artificial pupil and a lens correcting for accommodation. We have, however, observed that the above arrangement is a source of trouble for the subject and leads to confusion of the results much more serious than the one we observed later, when we gave up the artificial pupil and the correcting lens. Indeed, when the observer is adapted to complete darkness and the stimuli are at most twice the threshold stimulus, we are sure to exclude every chance of pupil contraction and the pupil will permanently maintain its maximum diameter.

The retinal region we investigated is region IV (near periphery) and our test area

was situated 15° temporally on the horizontal axis of the right eye. By varying the distance between the observer's eye and the test field and by varying the diameter of the latter, we have obtained circular retinal test areas varying from 2'12″ to 31'36″

TABLE II
Blue Light

Diameter of test area...........................		2'12″	3'30″	5'33″	8'51″	12'30″	14'	19'51″	31'30″
Relative values of S...........................		1	2.53	6.35	14.8	32.2	40.5	82.1	205
b (arbitrary units)	Observer M. R....	150	102°	45	20.8	7.77	7.97	3.11	1.20
	Observer F. A.....	163	61.2	26.6	11.4	5.06	4.86	2.30	0.71
$b \times S$ (relative values)	Observer M. R....	1	1.03	1.14	1.23	1	1.29	1.02	0.98
	Observer F. A. ...	1	0.95	1.04	1.04	1	1.20	1.16	0.90
	Mean values......	1	0.99	1.09	1.14	1	1.25	1.08	0.94
Coefficient x (mean values) computed from the relation $b \times S^x =$ constant..		—	1.01	0.95	0.96	1.00	0.94	0.98	1.01
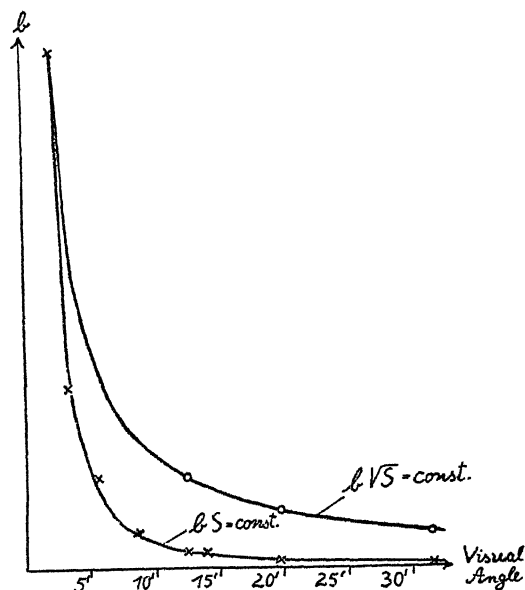
FIG. 7. o, threshold brightness for extremely red light (cones). ×, threshold brightness for the blue light (rods).

in diameter. We are now pursuing the same investigations with another apparatus, allowing for visual angles up to 8°.

Table II shows the relation between the threshold brightness b and the visual angles subtended by the test areas; Fig. 7 shows the average values for two

observers. It should be noted that the observed values correspond closely with a curve the analytical expression of which is: bS = constant. This is precisely Ricco's law and it is now demonstrated that it applies continuously from 2'12" to 31'36" and, perhaps, further on.

It was necessary to make sure that the thresholds measured by this method were independent of the stimulation time t. We proceeded to study this by seeking experimentally the value τ which is the point from which bt begins to increase. By using stimulations lasting noticeably less than τ (in fact we used $t = 8.3$ milliseconds for areas of 14' or more in diameter and $t = 20$ milliseconds for smaller areas), we made sure that the threshold values we had obtained were indeed independent of t.

The Spatial Law of Threshold in Peripheral Cone Vision

The relationship between threshold in peripheral cone vision and tested retinal area has been studied by Piéron, whose results did not allow him to formulate any law. The quantic and statistical theory of visual excitation enables us to predict this law. To do so, we have but to look up the histology of the retina.

In region IV, the number of cones per area unit is about $1/30$ of the number of rods (Østerberg). In this region, the number of rods belonging to the same ganglion cell is about sixty and there are also two cones linked to the same cluster of rods.

Our quasi-independent unit includes thus either one cone only or two cones contained in the same cluster. When there is a certain number of clusters stimulated by extremely red light, Piper's law must apply (see p. 274).

We measured the threshold for extremely red light for areas of 12'30" to 31'36" in diameter. Before the runs were made, there was a period of dark adaptation, 20 minutes long.

Fig. 6 shows the course of the photometric density of the red filter used, in relation to wavelength. From this graph we can see that every light of 679 mμ wavelength or less is practically absorbed by this filter and that the light it does transmit is chiefly composed of radiations the wavelengths of which are superior to 700 mμ. It should be noted here that the energy distribution of our light source has its maximum far beyond 700 mμ; so we are sure that our stimulus contains much less short-wavelength energy than it would seem from the graph of Fig. 6. Rod visibility curves (Hecht and Williams) allow us to show by computation that, in such conditions, only the cones must have been stimulated.

The important fact should be noted that every stimulation leading to a sensation was recorded by both subjects as "red light." Therefore we may state that *in peripheral vision of extremely red light the photochromatic interval is null*.

Fig. 7 shows our average results and Table III the individual results obtained from each observer. The measured values correspond remarkably well with a curve $b\sqrt{S}$ = constant. This is Piper's law. We have not been able to study areas smaller than 12'30" in diameter because of the insufficient luminosity of our source; a new apparatus is now being constructed, which will allow us to study very small areas, as well as areas several degrees in diameter.

The measurement of τ, in the case of peripheral cone vision, provides another important piece of information. Between 12'30" and 31'36" τ has been found to be practically constant, while in rod vision and for the same scale of diameters it varies from about 25 milliseconds to about 10 milliseconds. Now 120 milliseconds is precisely the value of τ which we found for the smallest area (2'12" in diameter) stimulated by blue light. This also proves that the liminal

TABLE III
Red Light

Diameter of test area		12'30"	19'51"	31'30"
Relative values of S		1	2.53	6.35
Relative values of $\sqrt{S}$		1	1.58	2.52
b (arbitrary units)	Observer M. R.	400	225	186
	Observer F. A.	143	98	47.4
$b \times \sqrt{S}$ (relative values)	Observer M. R.	1	0.90	1.17
	Observer F. A.	1	1.08	0.83
	Mean values	1	0.99	1.00
Coefficient x (mean values) computed from the relation $b \times S^x$ = constant		—	0.51	0.50

excitation of peripheral cones does take place because of the absorption of 2 quanta by a single cone or by two cones included in the same cluster of rods.

Liminal Brightness in Relation to Stimulation Time

Let us now compute the course and the numerical values of b for a given retinal area. For this purpose it is sufficient to know the probability that, among N quanta absorbed during the stimulation time t, two at least are separated by a time interval less than or at most equal to τ. The problem becomes one of computing the probability $P_{N,t,\tau}$ that among N points, chosen at random on a segment of a straight line, two at least are separated by an interval less than or at most equal to τ. We owe to Paul Lévy the generalized solution of this problem; a particular solution is

$$P_{N,t,\tau} = 1 - \left[1 - \frac{(N-1)\tau}{t}\right]$$

When $P_{n, t, \tau} = 0.5$—threshold condition—we may compute N. But as N is the *mean* number of quanta, we must take into account Poisson's law of small numbers. Indeed, when we consider a *single flash*, the number q of quanta actually absorbed may be less than, equal to, or more than N. We must therefore write:

$$P_{q, t, \tau} = 1 - \sum_{q=0}^{q=t/\tau} C_{q, N} \left[1 - \frac{(q-1)\tau}{t} \right]^q , \tag{1}$$
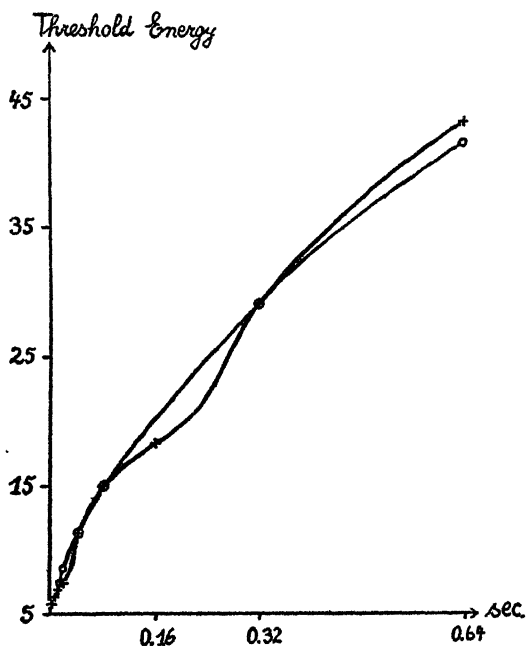
FIG. 8. ×, relative threshold brightness values in relation to stimulation time (1° area) (Graham and Margaria). O, computed curve with $\tau = 3.2$ milliseconds.

where the $C_{q, n}$ (Poisson's coefficients) express the probability that q quanta will be absorbed. Thus we may compute the ratio $bt/b\tau$ for any value of t.

When $t \leqq \tau$, the mean liminal flux carries less than two quanta. It seems convenient to take as the threshold condition 60 per cent positive answers. In this case, the mean liminal flux amounts almost exactly to two quanta (actually a two quantum flux corresponds to 59.4 per cent of positive answers).

Let us now consider the bt curve corresponding to the area 1° in diameter tested by Graham and Margaria (Fig. 8). If τ is given a value of 3.2 milli-seconds (its precise value does not appear from Graham and Margaria's exper-

iments but is included between 2.5 and 5 milliseconds), we may compute the theoretical curve by means of formula (1). It fits well with the general course of the experimental curve but we may distinguish in the latter three fragments of parabolic kind. There are two sharp upward movements: one at about 20 milliseconds, the other at about 200. Between 300 and 640 milliseconds bt seems to resume its ordinary course, as becomes obvious when we compare the theoretical and the experimental curves. One might think that these two sharp upward movements are due to errors in measurement, but this does not seem to be the case, for they are, on the contrary, systematic.

Indeed, the upward movement that takes place at about $t = 200$ milliseconds may be found also in the curves corresponding to areas 2' and 16' in diameter; moreover, it is also to be found in the curve expressing the results obtained by Piéron (Figs. 8 and 9). We shall discuss these systematic deviations at the end of this paper.

Ricco's Law, the Constant τ, and the Morphology of the Ganglion Cells

The fact that Ricco's law is valid in the retinal region considered, up to visual angles of 1°, makes probable the existence of ganglion cells with ramifications spreading at least as far as 300 μ: such a distance corresponds to a visual angle of 1°. Indeed, the histology of the retina shows that region IV is specially rich in giant ganglion cells which are perhaps nothing but a kind of parasol cell (Polyak). These cells are actually morphological units and their ramifications spread often farther than 350 μ. On the other hand, these ramifications overlap those of the neighboring giant cells. The figure 300 μ, computed from Graham and Margaria's psychophysiological measurements, fits, therefore, very well with the histological facts. Such a giant cell would then be what, in rod vision, we have called a "quasi-independent unit;" it would be linked to every smaller ganglion cell contained in the area circumscribed by its ramifications. We have been led to admit (Baumgardt) that the constant τ is a characteristic of the largest ganglion cell existing in the tested area; it should be remembered that the larger the stimulated area, the smaller the constant τ. But as we have observed that τ seems to decrease continuously when S increases, we must admit that there may be another explanation. Indeed, τ might depend on the mean distance between the two rods absorbing one quantum each, for we are aware of the existence of damped subliminal action potentials called "not propagated" action potentials, which, however, spread over a distance of about 1 mm. (Katz, Hodgkin). Therefore we can understand that the smaller the course of subliminal action potential generated by the absorption of one quantum, the greater is τ, for in such a case, the damping effect is less important than when the distance between the absorbing rods is long. At any rate, the solution of this problem requires a serious investigation of the course of τ in relation to S.

Deviation between Experimental Time Law and Theoretical Time Law

We have seen that the curves obtained by Graham and Margaria are of the same type as those which theory leads us to expect. However, there remains a systematic deviation, which becomes more important as the stimulated area becomes smaller. Let us consider Fig. 8, which expresses the relation between bt and t. The tested area measures 1° in diameter and the value of τ is between 2.5 and 5 milliseconds. What is the reason for these deviations between experimental and theoretical curves?

Indeed, the quand movement that takes 200 milliseconds is may be found also in the curves corresponding to areas 2' and 10' in diameter; moreover, it is also to be found in the curve expressing the results obtained by Piéron (Figs. 5 and 9). We shall discuss these systematic deviations at the end of this paper.

FIG. 9. O, relative threshold brightness values in relation to stimulation time, (16' area) (Graham and Margaria). ✕, the same (5'30" area) (Piéron).

We think that there are two reasons. First, the formula does not take into account the fact that the absorption of the two quanta may take place within the field of one of the many smaller ganglion cells existing in the stimulated area. Therefore, when t is less than 20 milliseconds, the actual probability P is definitely greater than the computed probability P. Indeed, Fig. 9 shows that for 16' area bt is constant when t is less than 20 milliseconds. Secondly, when t increases, the mean number N of absorbed quanta also increases and q, the number of quanta actually absorbed, more and more often is greater than 2. When, in a single test, the time interval between the absorption of the two first quanta is greater than τ, there is no propagated action potential, but owing to the local excitation caused by the absorption of the first quantum, the

ganglion cell concerned must go through a relatively refractory state which prevents the raising of a propagated action potential *even if the absorption of a third quantum follows the absorption of the second one within a time interval not longer than τ*. It will therefore be necessary for the third quantum to follow the second one very closely or, perhaps, for a fourth one also to be absorbed. In any case, *bi* will then increase more rapidly than we should expect from the relation shown in equations (1).

The second upward movement (at about 200 milliseconds) may then be ascribed to the refractory state of the smallest ganglion cells. Indeed, inside a 16' area, there are about twenty clusters of rods, and the data of Graham and Margaria show that for smaller areas consisting of a part of a single cluster, there is at about 200 milliseconds a sudden upward movement of *bi* which may be explained by the above mechanism. It is not surprising to find the same phenomenon on the curve corresponding to the 16' area (see Fig. 9), and in Fig. 8 (1' area) we may observe it once more.

It seems that the quantic and statistical theory cannot by itself allow the prediction of these effects. Indeed these sudden upward movements are due to the fact that the more subliminal action potentials there are, the more the ganglion refractory state is reinforced; they are *nervous phenomena*. This mechanism which actually produces a *nerve adaptation* may give us a clue to the important problem of why, a long time before the mechanism of photochemical adaptation begins to come into play, the sensation level increases much more slowly than the brightness. The analysis of the measurements of differential threshold, made by König and Brodhun, shows that the sensation level increases approximately linearly with brightness when brightness is very faint; then it becomes proportional to the square root of the brightness, then proportional to its cubic root, and finally proportional to its logarithm. It is in this last range of brightness that the sensation level follows the mass action law, essential basis of any photochemical theory of vision. But when brightness increases still further, the logarithmic law must be replaced by another law which determines a slower increase of the sensation level; and finally, when the brightness has become such that each purple molecule just recombined is immediately decomposed again by a quantum, the sensation level will have reached its maximum.

We are far from being able to follow these mechanisms mathematically. But we believe that it is necessary to distinguish on the one hand the phenomena due to the quantic and random character of the stimulating light, and on the other hand, those due to nervous adaptation (retinal and no doubt cortical too), and to photochemical adaptation. Thus, we may hope, thanks to mathematical analysis, to come to a new theory of vision lacking all speculative character and using no arbitrary parameters. Such a theory might advantageously replace the actual photochemical theories, which by means of a certain number of parameters carefully chosen have so far vainly tried to express by a single

formula what actually seems to be the result of several essentially different mechanisms.

SUMMARY

1. The photochemical theories of vision cannot provide a valid interpretation of the facts over the whole range of brightness. The fact that liminal excitation is increased by the absorption of a very small number of quanta, each absorbing rod receiving a single quantum, excludes the intervention of the mass action law which is the basis of all photochemical theories.

2. Owing to the quantic structure of light and to the random distribution of quanta in a faint light pencil, there must exist numerical relations between the threshold energy on the one hand and the size of the retinal area stimulated and the stimulation time on the other, whatever may be the inner mechanism of liminal excitation. When taking as a basis Van der Velden's experimental results, viz. that two quanta absorbed during a certain interval of time are sufficient to raise threshold excitation, the probability calculus enables us to compute the course of threshold energy in relation to the stimulation time and to the stimulated retinal area. No arbitrary parameter is needed to do so; the only constant to be used is found by experiment.

3. The quantic and statistical theory of visual excitation that we put forward in the present paper enables us to predict the validity of Ricco's law within what we call a "quasi-independent unit" and the validity of Piper's law within a test area made up of a certain number of such units. This theory does not correspond exactly with Piéron's law for foveal threshold in relation to the size of the stimulated area, but the deviation is probably due to an artefact; viz., the action of the micronystagmus.

4. Experiment proves that in region IV of the retina, 15° temporally from the fovea of the right eye of two observers, Ricco's law applies strictly in rod vision from 2'12" to 31'36" and, perhaps, further on.

5. In the same region, from 12'30" to 31'36", Piper's law applies strictly in cone vision of extremely red light.

6. In peripheral vision with extremely red light the photochromatic interval has been found to be null.

7. Our theoretical interpretation of the term "quasi-independent unit" fits well with the histological data of the retina.

8. Numerical deviations of the theoretic time law of threshold intensity from the empirical course may be due to the existence of a relative refractory period of the ganglion (or bipolar) cells. This mechanism would be a sort of instantaneous adaptation of nervous elements and would explain the fact that the sensation level increases very much slower than the brightness level, in a range of the brightness scale where the photochemical adaptation cannot account for this phenomenon.

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ABNORMAL PROTOPLASMIC PATTERNS AND DEATH IN SLIGHTLY HYPERTONIC SOLUTIONS

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PLATES 5 TO 7

A variety of experiments indicates that the life processes of the cell depend to a large extent on the behavior of the protoplasmic surface. It is therefore important to utilize all available means to follow changes in this surface.

It is of much interest from this point of view to find that alterations in the protoplasmic surface of *Nitella* due to plasmolysis are accompanied by striking changes in the chloroplasts. Such changes have apparently not been seen in other cells but in *Nitella* they are very obvious. Slightly hypertonic solutions of sugars and of electrolytes may cause contraction of the chloroplasts. The clear spaces between them enlarge in certain regions and assume characteristic patterns (Figs. 1 to 6). Such changes are irreversible and are soon followed by death.

A study of this behavior may throw light on the nature of the protoplasmic surface and on the properties of protoplasmic gels as well as on the process of death. An understanding of the mechanism involved may help to explain the action of hypertonic solutions in other cases as, for example, in the artificial parthenogenesis of marine eggs.

The protoplasm in which these changes occur consists of a layer not over 15 microns thick. The outer portion of the protoplasm is a gel in which the ellipsoid chloroplasts are arranged in longitudinal rows (Fig. 1). As we focus down through the cell to the vacuole we encounter only a single layer of chloroplasts so that an incident ray of light coming from above never passes through two chloroplasts in succession before reaching the vacuole. The inner part of the protoplasmic layer is liquid and normally shows constant streaming. Inside this layer of protoplasm is the large central vacuole filled with sap.

When cells[2] are placed in a slightly hypertonic solution of sucrose (0.25 to

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[1] This is shown by the behavior in the centrifuge where the outer part of the protoplasm may split up into long straight rods, each containing a single row of chloroplasts. Curved structures of this sort are seen in Fig. 5.

[2] The observations were made on *Nitella flexilis*, Ag. The cells were freed from neighboring cells and observed at once or kept in the laboratory in Solution A (*cf.* Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1933–34, 17, 87) at 15°C. ±1°C. An hour before use the temperature was raised to about 25°C. Cells about 8 cm. in length were used.

Some cells were resistant and did not develop splits as the result of slight plas-

0.4 M) so that slight plasmólysis[3] occurs in 5 minutes or less we usually see immediate changes in the ellipsoid chloroplasts. They shrink somewhat and their shape may become more nearly spherical; eventually they may round up into spheres[4] whose diameter is less than that of the smallest diameter of the original ellipsoid. This evidently involves a loss of volume.

These alterations increase the amount of clear space between the longitudinal rows of chloroplasts. A further increase may be caused by the displacement of chloroplasts toward the interior of the cell so that they come to lie on a different level. We may then encounter, as we focus down into the cell, 2 or 3 successive layers of chloroplasts instead of a single layer as is normally the case. This occurs more often in the later stages of the process.

Soon after the contraction of the chloroplasts begins we see that in certain places the narrow clear spaces between the longitudinal rows of chloroplasts become wider (Figs. 2 and 3). Since these widened clear areas look like splits this term will be used for convenience in description.

The splits appear as clear areas entirely free from chloroplasts (Figs. 2 to 6); they run lengthwise, tapering off at both ends.[5] In a single cross-section of the cell there may be as many as a dozen such splits. Some of them steadily increase in size but their development does not follow a regular pattern. Several small splits may appear at about the same time in the same region, some developing much faster than others (Fig. 5). Sometimes there is only a single split in a given region and this may increase so as to occupy nearly half of the circumference of the cell.[6] The formation of splits may thus involve a considerable increase in the amount of clear space between the longitudinal rows of chloroplasts.

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molysis. In former experiments with plasmolysis (Osterhout, W. J. V., *J. Gen. Physiol.*, 1943–44, **27,** 139) all the cells were resistant and recovered if the plasmolysis had not gone too far. In these cells no splits were observed.

[3] In some cases considerable loss of water results in a flattening of the cell (change in shape of the cross-section) rather than in a retraction of the protoplasm from the cellulose wall.

[4] It might be suggested that the appearance of shrinkage is brought about by a shift in the position of the chloroplast by which its long axis ceases to be parallel to the surface of the cell. This does not appear to be the case when starch grains in the chloroplast enable us to see differences in the opposite ends of the chloroplast.

[5] These are quite different from the irregular areas devoid of chloroplasts which sometimes appear in cells not subjected to plasmolyzing solutions and which may possibly result, in part at least, from manipulation in collecting and separating the cells. But such areas sometimes develop along with the splits.

[6] Even where there is little contraction of the chloroplasts the clear space between some longitudinal rows may diminish as the splits increase so that the clear space in the splits increases at the expense of the clear space between rows where no splits exist.

The splits may continue to develop for an hour or more. When the process reaches a certain stage death ensues, as shown by the entrance of acid fuchsin,[7] and by the exit of chlorides[8] (as demonstrated by adding $AgNO_3$ to the external solution).

The development of splits appears to be irreversible from the start. If the cell is transferred to distilled water as soon as the splits begin to appear they may continue to develop very much as though no change had been made in the external solution.[9] When the plasmolyzing solution is replaced by water the protoplasm expands but the chloroplasts do not, and this creates more clear space which is incorporated into the splits. But in many cases this alone does not appear adequate to explain the increase in the size of splits after the cell is returned to water and this increase may be aided by shrinkage of the chloroplasts and their displacement toward the interior of the cell.

If the cell shows protoplasmic motion just before it is returned to water the motion usually stops soon after the return to water and death soon follows, as shown by the entrance of acid fuchsin and the exit of chlorides. The turgor may be temporarily regained after the return to water but it soon disappears. The protoplasm usually contracts soon after this (false plasmolysis) and may shrink to half its normal diameter. In certain cases, however, if the exposure to the plasmolyzing solution has been less than a minute and retraction of the protoplasm occurs without any contraction of the chloroplasts or formation of splits the cell may live for some hours after the return to water.

In most cases splits do not form in solutions of sucrose which are not able to produce evident plasmolysis.

In some cases the formation of splits begins just before the retraction of protoplasm from the cellulose wall (the retraction may occur only in certain parts of the cell but the splits may appear everywhere.

With these facts in mind we may consider the conditions under which the splits arise. The outer layer of the protoplasm is a stiff gel in which the chloroplasts are arranged in longitudinal rows, each row in a rod of gel.[10] When plasmolysis occurs it is not surprising that as these rows become displaced the longitudinal arrangement of the chloroplasts is preserved. The shrinkage of

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[7] The acid fuchsin (National Aniline Co.) is used in 1 per cent solution in phosphate buffer (at pH 8) which has a cationic concentration of 0.006 M. If the dye is merely dissolved in water the pH is about 2.6 which is injurious.

[8] Cf. Osterhout, W. J. V., J. Gen. Physiol., 1922–23, 5, 709.

[9] If plasmolysis begins without formation of splits and if the cell is then placed in water the protoplasm may expand without development of splits but usually the cell does not live long after this. During this expansion of the protoplasm the normal motion may continue for a time.

[10] This is shown by subjecting the cells to centrifugal force which causes the protoplasm to split up into rods each of which contains a single row of chloroplasts.

the chloroplasts adds to the clear space between the rows but we do not know why the clear space increases chiefly in certain places to form splits.

It might be suggested that splits are due to movement of water, as, for example, when the plasmolyzing solution is replaced by water and there is a sudden rush of water into the protoplasm followed by the development of splits. But when this mechanical disturbance is lessened by transferring the cell from the plasmolyzing solution in gradual steps[11] splits appear as before.

It is evident, however, that if a mechanical disturbance of the protoplasm is great enough it may produce splits. For example, when a cell is placed on a slide without a coverglass we may cover a region A at one end with water separated by a vaseline barrier from the rest of the cell[12] (called B). If we place 0.5 M sucrose at A, water enters at B and a rush of sap in the vacuole toward A is observed. After half an hour the 0.5 M sucrose at A is replaced by water. We then see a rush of sap in the vacuole from A to B. This occurs because the previous exit of water at A has left behind osmotically active substances which cannot pass out through the protoplasm so that the osmotic pressure in the sap at A has increased.[13] When the sucrose at A is replaced by water the latter enters at A, rushes along the vacuole[14] to B and passes out at B. The splits which sometimes develop at B[15] might be attributed to the mechanical disturbance of the violent outgoing current at B.

In some cases splits develop at A but in order to produce splits and irreversible contraction of the chloroplasts the ingoing current must be more violent than that described in a former paper[16] where no splits were produced by the ingoing current and the contraction of the chloroplasts could be reversed by an outgoing current.

In experiments lasting several days in solutions of sucrose too dilute to produce plasmolysis cells do not live so long as those kept in distilled water, indicating toxic action due to the sucrose solution (or to organisms growing in it); but this does not appear to play a part in plasmolytic experiments since in the dilute solutions death occurs without production of splits.

Turning now to the chloroplasts it may seem surprising that the contraction

[10] E.g. from 0.4 M sucrose to 0.25 M, then to 0.1 M, then to 0.05 M, and then to water.

[12] A and B are each about 30 mm. long. *Cf.* Osterhout, W. J. V., *J. Gen. Physiol.*, 1946–47, **30**, 439.

[13] The hydrostatic pressure does not become greater at A than at B but the osmotic pressure may do so. *Cf.* Osterhout, W. J. V., *J. Gen. Physiol.*, 1946–47, **30**, 439.

[14] If the sucrose at A is replaced by water the rush toward B may be so violent that some of the chloroplasts near the vaseline barrier in A may be dislodged and carried along the cell toward the end wall.

[15] The ingoing current causes the chloroplasts to contract as described in a former paper (Osterhout, W. J. V., *J. Gen. Physiol.*, 1946–47, **30**, 229); they expand again in the outgoing current.

[16] Osterhout, W. J. V., *J. Gen. Physiol.*, 1946–47, **30**, 229.

of the chloroplasts which accompanies plasmolysis occurs in an outward current of water because it was stated in a former paper[19] that contraction occurs in an ingoing but not in an outgoing current. But in the former experiments only part of the cell was covered with the sucrose solution and water could enter freely into the rest of the cell (which was covered with water) and there was no indication of plasmolysis at any point. In the present experiments the plasmolyzing solution covered the entire cell and the resulting plasmolysis appears to be necessary for the production of splits except when the outgoing current is violent as described below.

It should be noted, however, that contraction of the chloroplasts may occur before the loss of water becomes great enough to produce visible retraction of the protoplasm from the cellulose wall.

In this connection the following experiment is of interest. A region at one end of the cell about 25 mm. long, designated as A, is covered with 0.5 M sucrose, separated by a vaseline barrier from the rest of the cell, B, which is covered with water. Water then enters at B, there is a rush of liquid in the vacuole toward A, and water comes out at A. If the outgoing current is strong enough[17] splits may occur at A and at their edges there may be some contraction of chloroplasts extending to 3 or 4 rows on each side of the split. These chloroplasts appear to be in much the same condition as the small rounded chloroplasts on each side of the "white line" in normal cells. These white lines resemble splits in some respects. They occur under normal conditions at the two edges of an imaginary plane which runs lengthwise through the center of the cell and separates the protoplasmic current flowing in one direction from the current in the opposite direction. Along the two lateral edges of this plane we see two white lines, the well-marked clear spaces devoid of chloroplasts on opposite sides of the cell, running along its entire length. We usually see particles moving in opposite directions on opposite sides of the white line.[18] As a rule none of the particles crosses the white line.

It is an interesting fact that when splits are produced by sucrose the normal white lines usually widen.

If we use a higher concentration of sucrose (0.5 M or 0.6 M) in this experiment water may come out at A faster than it can be supplied through the vacuole from B and as a result there may be slight plasmolysis at A.

Results resembling those described here were obtained in preliminary experiments with glycerol, glucose, mannitol, raffinose, NaCl, CaCl₂, and sea water.

[17] To ensure this it may be necessary to increase the concentration of the sucrose. If it is raised to 0.5 M or 0.6 M plasmolysis may occur at A even though water is entering at B.

[18] Owing to the slightly spiral course of the longitudinal rows of chloroplasts the white lines on opposite sides of the cell appear to cross at certain places as we focus down through the cell.

[19] See Osterhout, W. J. V., J. Gen. Physiol., 1920, 2, 30.
[20] Osterhout, W. J. V., Proc. Nat. Acad. Sci., 1916, 2, 237.

A variety of solutions too dilute to produce plasmolysis may cause death without producing splits. Among these are solutions of $HgCl_2$, iodine, acetic acid, oxalic acid, and hexylresorcinol.

If the effects hitherto described were due merely to loss of water (without retraction of the protoplasm from the cellulose wall) they might be expected when the loss of water is brought about by evaporation. In order to test this cells were placed on slides without water and watched while evaporation went on. As a rule only one or two large splits developed but there was little or no retraction of the protoplasm and no appearance of the characteristic pattern of numerous small splits.

The experiment was varied by removing adhering drops of water from the cell by means of filter paper and then covering it with a thin layer of light paraffin oil (for medicinal use). A slow evaporation of water then occurred through the oil. This produced much the same effect as evaporation in air.

DISCUSSION

These experiments reveal a novel set of reactions. Slight plasmolysis causes the chloroplasts to shrink and shift so that the space between them increases and assumes a characteristic pattern with numerous clear areas running lengthwise in the cell.

The process is irreversible. Once started it continues even when the plasmolyzing solution is replaced by water. Eventually the sap in the vacuole passes out through the dying protoplasm which may become a compact elongated mass with no appearance of vacuole.

The contraction of a chloroplast involves a loss of volume[19] but this is not necessarily the case with the protoplasm as water escapes from the vacuole.

Whether the shift in the position of the chloroplasts is due to changes in the chloroplasts or in the surrounding protoplasm or in both is not clear. It is possible that local changes in the thickness of the gel portion of the protoplasm might push the chloroplasts into the pattern seen in the splits. But it is also possible that this pattern might arise as the result of an active movement of the chloroplasts. Active movements of chloroplasts occur in many plants but are usually determined by the direction and intensity of the incident light.

It might be suggested that the normal arrangement of the chloroplasts is due to a mutually repulsive action. In the red alga *Griffithsia*[20] this may be the case for when all the chloroplasts are collected in one end of the cell under the action of centrifugal force they may return to their normal positions when the centrifugal force ceases to act. We do not know to what extent mutual repulsion plays a part in the normal arrangement of the chloroplasts in *Nitella* or in the production of splits.

[19] See Osterhout, W. J. V., *J. Gen. Physiol.*, 1946–47, **30**, 229.
[20] Osterhout, W. J. V., *Proc. Nat. Acad. Sc.*, 1916, **2**, 237.

In seeking the cause of these phenomena we may consider (1) chemical effects, and (2) mechanical action.

1. It does not seem that chemical effects due to loss of water are the chief cause of the numerous small splits since loss of water can occur without producing the characteristic pattern seen in plasmolysis. For example, when a cell is placed on a slide and water is removed by means of filter paper and the cell is then observed while evaporation goes on we usually see a single large split and the cell may flatten to a thin ribbon, but there is no separation of the protoplasm from the cellulose wall and no development of the characteristic small splits resulting from plasmolysis in hypertonic solutions.

It seems improbable that the splits are caused by the chemical action of sucrose for they are not produced by prolonged exposure to solutions too dilute to plasmolyze, although death may ensue after an exposure of several days to such solutions.

2. It would therefore seem probable that the splits are due chiefly to mechanical causes. Apparently splits are associated with retraction of the protoplasm from the cellulose wall although it is not necessary that the retraction should occur at the exact point where the splits occur and the splits may come a little sooner than the visible retraction. It seems possible that injury initiated at one point may spread along the cell as in the marine alga *Griffithsia*.[20]

In harmony with this view is the fact that an ingoing current of water (p. 294) or an outgoing current (p. 294) may, if sufficiently strong, produce splits. This might well be a mechanical effect although chemical changes due to the removal of substances by the current of water are not excluded.[19]

On this view the loss of water would be important chiefly as causing the mechanical changes accompanying retraction of the protoplasm from the cellulose wall.

How can the retraction of the protoplasm from the cellulose wall produce injury? It must be remembered that the non-aqueous film which covers the surface of the protoplasm is a very delicate structure too thin to be visible under the microscope. The experiments indicate that the normal processes of the cell depend on the integrity of this film. It would seem that such a structure might be easily damaged in the process of protoplasmic retraction. And it seems possible that such injury may occur even without visible retraction of the protoplasm if the cellulose wall is penetrated by delicate threads of protoplasm such as have been described for various plant cells.[21] Some of these extensions are just at the limit of visibility. If there are protoplasmic extensions in *Nitella* as attenuated as the non-aqueous surface layer of the protoplasm they may be invisible. If the process of retraction begins with the withdrawal of such extensions from the cellulose wall irreversible changes may occur before any visible

[21] *Cf.* Küster, E. Die Pflanzenzelle, Jena, G. Fischer, 1935, 103.

retraction of the main body of protoplasm takes place. Such irreversible injury might conceivably involve production of splits. Slight plasmolysis at the top or bottom of the cell might escape detection.

It is of interest to note that if we accelerate the process sufficiently by using a higher concentration of sucrose so as to produce rapid retraction of the protoplasm there may be no appearance of splits and protoplasmic motion may continue much longer than with slight plasmolysis. This may be true even when the cross-section of the protoplasm has been reduced by 25 per cent in rapid plasmolysis. But when such cells are replaced in water splits occur and death follows.

If the splits result from mechanical disturbance of the non-aqueous film at the outer surface of the protoplasm it would seem that slow retraction of the protoplasm is more effective than rapid and expansion is effective as well as retraction (as seen when the cell is returned to water after plasmolysis). But the rapid plasmolysis usually withdraws more water and this may play a part.

It might be suggested that the changes observed in hypertonic solutions bear some resemblance to those seen in syneresis.

The injurious effects of plasmolysis are shown by contraction of the chloroplasts and by the formation of splits. But the formation of splits seems to be more significant since, as shown in former papers,[22] contraction is a reversible process when brought about by lead acetate or by an ingoing current of water.

These effects on *Nitella* are of interest in connection with the action of hypertonic solutions in artificial parthenogenesis, particularly in view of Loeb's suggestion that this involves an injurious action which is checked before it has gone too far.

The processes leading to the formation of splits throw additional light on properties of protoplasmic gels which have recently been discussed[16, 23] in connection with *Nitella* and *Spirogyra*. Further studies in this field are desirable.

It would seem that if we understood the mechanism which produces splits we should have a much better picture of the death process and this in turn would throw light on the mechanism of life processes.

SUMMARY

Some interesting properties of protoplasm are revealed when slightly hypertonic solutions of sugars or of electrolytes are applied to *Nitella*.

The chloroplasts contract and the space between them increases and forms a characteristic pattern consisting of clear areas extending lengthwise along the cell and tapering off at both ends.

The development of these areas is irreversible from the start. If the cell is

[22] Osterhout, W. J. V., *J. Gen. Physiol.*, 1945–46, **29**, 73; 1946–47, **30**, 229.
[23] Osterhout, W. J. V., *J. Gen. Physiol.*, 1945–46, **29**, 181.

returned to water after plasmolysis begins these areas continue to enlarge in much the same fashion as when no change is made in the external solution. The cell soon dies whether returned to water or left in the plasmolyzing solution. Similar results are obtained with other sugars, with NaCl, CaCl$_2$, and sea water.

Similar reactions are also brought about by strong ingoing or outgoing currents of water. This suggests that mechanical action may be chiefly responsible for the result and this idea is in harmony with other facts. It seems possible that the retraction of the protoplasm from the cellulose wall may disturb the delicate non-aqueous film which covers the outer surface of the protoplasm and thus produce injury. Such an effect might take place even without visible retraction if the injury occurred in protoplasmic projections extending into the cellulose wall.

A study of this behavior may throw light on the nature of the protoplasmic surface and on the properties of protoplasmic gels as well as on the process of death. An understanding of the mechanism involved may help to explain the action of hypertonic solutions in other cases as, for example, in the artificial parthenogenesis of marine eggs.

EXPLANATION OF PLATES

PLATE 5

FIG. 1. Normal cell showing the ellipsoid chloroplasts arranged in longitudinal rows (in many cells these rows follow a slightly spiral course).

When the microscope is focussed below the center of the chloroplast (as seen in the middle of Fig. 1) the chloroplasts appear darker than the spaces between them because they consist of more highly refractive material than the substance lying between them. In this position of the microscope the chloroplasts seen at the top and bottom of the figure are in a lower plane than those seen in the middle of the figure so that the plane of the focus is above their centers and consequently they appear lighter than the spaces between them. This difference in the position of the chloroplasts is due to the curvature of the surface of the cylindrical cell. ×190.

FIG. 2. In several places the clear space between the longitudinal rows of chloroplasts has widened to form clear areas running lengthwise and tapering off at both ends; these are called for convenience "splits."

The figure shows several stages in the development of splits.

Several small clear circular areas surrounded by heavy dark lines are due to spherical ,bodies which are commonly found in the sap.

Plasmolysis is shown at the bottom and to a much less extent at the top (extreme left). ×120.
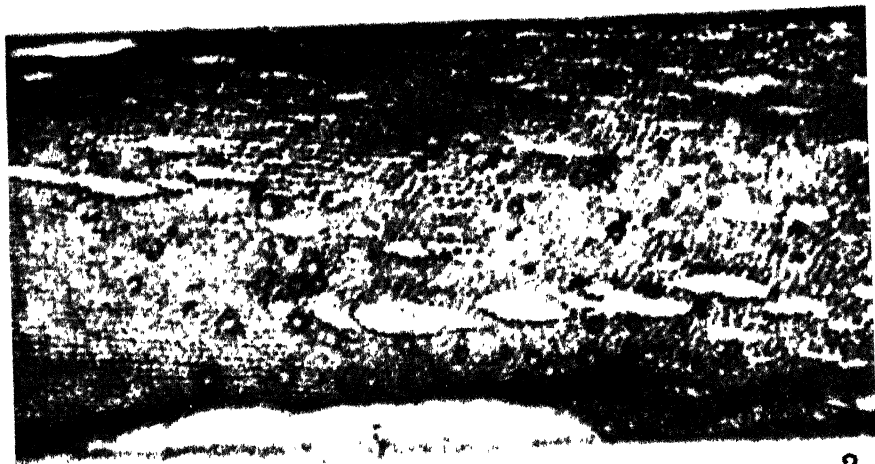
(Osterhout: Abnormal protoplasmic patterns)

FIG. 3. Small splits at the top, .arger ones at the bottom.

The chloroplasts are contracted a·d in some cases rounded up to form spheres. Slight plasmolysis.   ×170

FIG. 4. Well developed splits separa⁺ed in some places by single rows of chloroplasts.   Plasmolysis is shown at top and bottom.   ×200

3

4
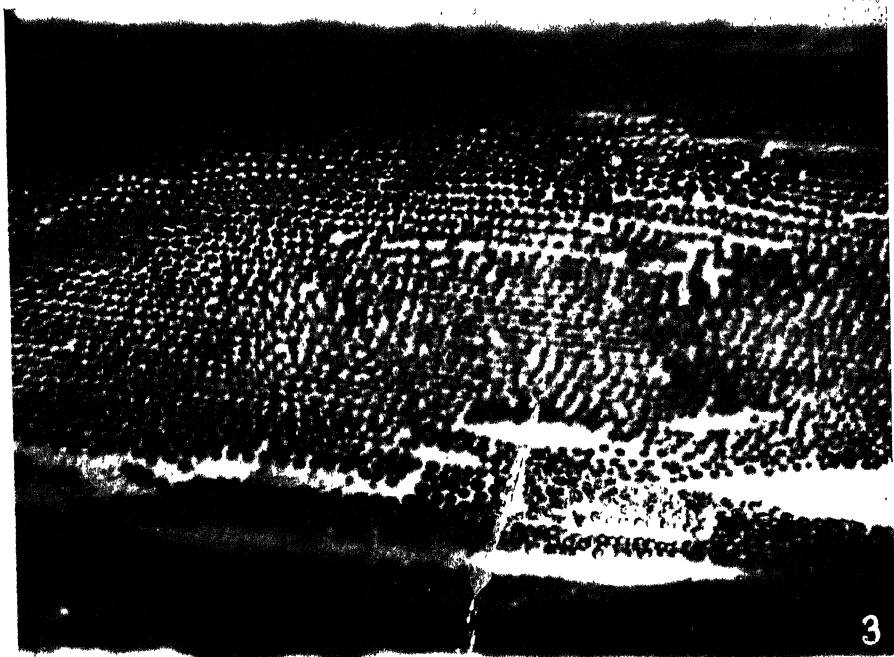
FIG. 5. Splits over the entire cell. In several places single rows of chloroplasts separated off. Plasmolysis is shown in lower left hand corner. In the lower right hand corner a large clear space with a sphere with a light center and a clear zone around it: such spheres often appear in the vacuole.   ×180.

FIG. 6. Very large splits. Each of these began as a very small split such as is visible in Fig. 2, upper right hand corner.   ×190.

5

6
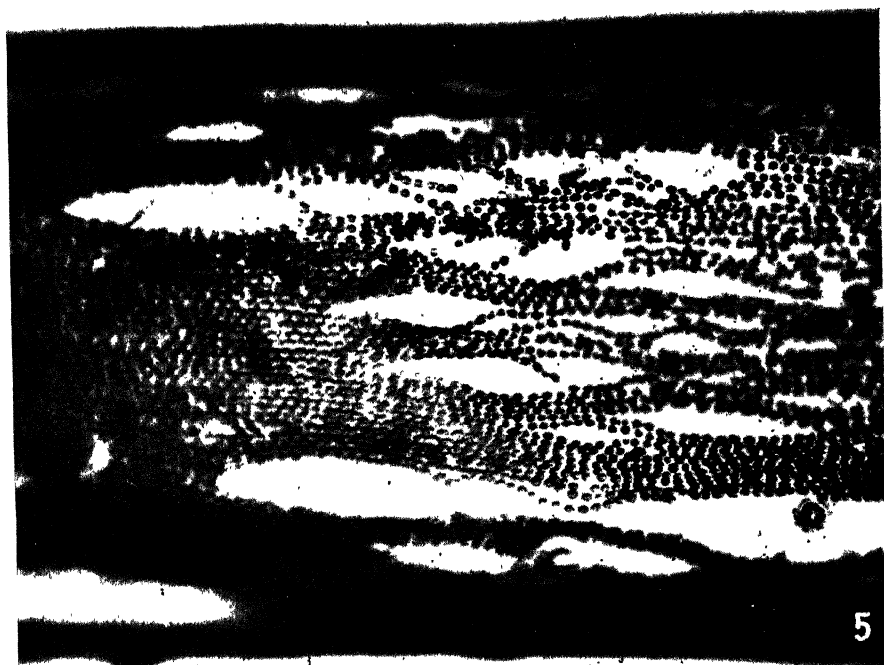
THREE-STAGE ANALYSIS OF BLOOD COAGULATION*

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Ever since Morawitz published his classical review in 1905, it has been the custom to divide the blood-clotting process into two phases, or stages: (1) Prothrombin is converted to thrombin in the presence of calcium ions and certain factors derived from platelets and tissues. (2) Under the influence of thrombin, soluble fibrinogen is transformed to the insoluble fibrin.

This formulation was put forth as tentative, and it was generally appreciated that a two-stage theory could not give an adequate explanation of the mechanism. Several attempts were made to construct a three-stage framework on which further detail might be built. Morawitz (1904) suggested that prothrombin was first acted upon by thrombokinase, then by calcium. In somewhat similar fashion, Howell (1935) believed that cephalin freed prothrombin by removing heparin from it; then the prothrombin was activated directly by calcium. The 1940 schema of Wöhlisch postulated the conversion of prothrombin I to prothrombin II. Bordet (1919) had proposed that prothrombin must be "unmasked" in order to be readily convertible to thrombin; and his experiments had shown with particular clarity that some time-consuming reaction did, in fact, precede the development of thrombin. However, his data did not prove the assumption basic to all these three-stage theories, namely that the preliminary reaction was concerned with some preparatory change in the status of prothrombin.

Meanwhile another idea had been growing. Mellanby (1909) interpreted some of his data as indicating that serum contained an inactive form of thrombokinase. Closer to the point was the demonstration by Dale and Walpole (1916) that fowl plasma contained an inactive form of thrombokinase, from which active thrombokinase could be released by treating the plasma with chloroform or trypsin. The concept of a plasma precursor of thrombokinase was embodied in a three-stage theory of blood coagulation by Lenggenhager in 1936; and the proposed mechanism was explicitly diagrammed by him in 1940, in principle, as follows:

1. Prothrombokinase → thrombokinase in the presence of calcium.
2. Prothrombin → thrombin   in the presence of calcium and thrombokinase.
3. Fibrinogen → fibrin   in the presence of thrombin.

Lenggenhager reported that prothrombokinase could be precipitated with a euglobulin fraction from platelet-free citrated plasma. He further stated that prothrombokinase was very labile and tended to become active even when gentle methods were used to precipitate the globulins. These observations were soon confirmed by Widenbauer and Reichel (1942).

The new three-stage theory offered Astrup (1944) a means of explaining the striking but enigmatic phenomenon of autocatalysis in blood coagulation. It had been known since the work of Arthus (1901) that thrombin was produced slowly, at first, then with increasing rapidity during the coagulation of whipped whole blood. Although by itself this observation might have been explained on the basis of two or more consecutive reactions, later work left little doubt that an autocatalytic or chain reaction was actually involved. Experiments described by Gratia (1922) and Fischer (1935) revealed that by serial seeding, an inciting agent for coagulation could be propagated through a series of otherwise stable samples of plasma. In quest of the underlying mechanism, Astrup searched for some evidence that thrombin catalyzed the activation of prothrombin, but his results were entirely negative (1939, 1944). He thereupon suggested that the autocatalytic effect was probably concerned with the activation of prothrombokinase, and indicated the chief impediments to further inquiry—the substances involved were labile and a reliable method of investigation had not been found.

The method of analysis described below was developed over a period of years, beginning with a study made in 1936 by Hellerman, Milstone, and Carnes. At that time it was found that the kinetics of prothrombin activation in crude preparations could not be described as a simple autocatalytic phenomenon, and that thrombin did not directly accelerate the activation of prothrombin. These unpublished findings suggested the same conclusions later reported by Astrup, and led to similar technical difficulties. The difficulties have now been overcome or circumvented sufficiently to allow the activation of prothrombokinase to be followed in a direct, quantitative manner.

The guiding idea in this work has been that if, indeed, the coagulation mechanism does embrace three distinct reactions, then it should be possible to carry out these primary reactions consecutively in separate test tubes. Moreover, as this goal was approached, new quantitative techniques should become feasible, and it should become easier to associate a given special effect, such as autocatalysis, with a particular primary reaction. Finally, it seemed of manifest importance to isolate and define the elusive "first" reaction, because the activation of prothrombokinase would naturally be a critical point in the physiologic control of the coagulation system.

Materials and Methods

*Veronal-Buffered Saline—pH 7.4.—*200 ml. 0.1 M sodium diethylbarbiturate
plus 144 ml. 0.1 M HCl
plus 0.9 per cent NaCl to 1,000 ml.

Stored in the refrigerator. The stock 0.1 M sodium diethylbarbiturate was also stored cold.

0.0275M Ca.—25 ml. 0.11 M $CaCl_2$ plus 75 ml. veronal-buffered saline.

0.0025M Ca.—10 ml. 0.0275 M Ca plus 100 ml. veronal-buffered saline.

BaSO₄ Suspension.—Prepared by the method of Tanturi and Banfi (1946) except that the $BaSO_4$ was collected and washed on a Büchner funnel.

Acetate Mixture for Prothrombin Preparation.—699 ml. 4 M sodium acetate plus 69 ml. glacial acetic acid. Glass electrode readings:

Undiluted,   pH 5.45.

Diluted tenfold pH 5.00.

Buffer for Fibrinogen—pH 7.4.—67 ml. 0.1 M Na_2HPO_4.

13.4 ml. 0.1 M HCl.

100 ml. isotonic potassium oxalate (0.1272 M).

0.9 per cent NaCl to 1,000 ml.

Fibrinogen.—650 mg. bovine fraction I (Armour) was dissolved in 50 ml. buffer. 17 ml. saturated ammonium sulfate was added slowly. 15 minutes later the mixture was centrifuged lightly for 5 minutes, and the precipitate was immediately stirred thoroughly with 200 ml. 0.25 saturated ammonium sulfate. The residue was collected by centrifugation and dissolved in 75 ml. buffer. This fibrinogen preparation was stored at −17°C. Before use, it was thawed in a water bath at 30°C., whereupon the fibrinogen promptly and completely dissolved.

Frozen Euglobulin.—The source material for all plasma derivatives other than fibrinogen was a frozen euglobulin precipitate obtained from Armour and Co., of Chicago, through the cooperation of Dr. J. B. Lesh. Bovine blood was citrated, and the cells were removed by Sharples centrifugation. The plasma was diluted with ten volumes of cold tap water and the pH brought to 5.1 by addition of 1 per cent acetic acid. After settling overnight in the cold the supernatant fluid was discarded and the precipitate was shipped by air express, packed in dry ice. This material was still usable for the present type of work after a year's storage at −17°C. although even when first received, it contained some fibrin. 45.4 gm. represented 1 liter of citrated plasma.

Heated Globulin.—6 gm. Armour euglobulin was triturated with 40 ml. veronal-buffered saline and the pH brought back to 7.4 by cautious addition of 0.1 N NaOH. The undissolved material was removed by centrifugation, the pH readjusted to 7.4, if necessary, and the fluid transferred to four 12 ml. conical centrifuge tubes. The tubes were then kept in a 51°C. water bath for exactly 16 minutes and in cold tap water for 5 minutes. They were then allowed to stand at room temperature for ½ hour, during which the degree of flocculation increased. The heat-coagulated fibrinogen was then removed by centrifugation.

Prothrombokinase.—30 ml. heated globulin was mixed with 9 ml. $BaSO_4$ suspension in a 50 ml. centrifuge tube. The tube was stoppered with a clean rubber stopper and inverted at intervals for 20 minutes to keep the $BaSO_4$ suspended. The $BaSO_4$ was removed by centrifugation, and the adsorption procedure was repeated on the supernatant fluid, using 9 ml. of fresh $BaSO_4$ suspension. After 20 minutes, the $BaSO_4$

was centrifuged down, and the supernatant fluid was added to 270 ml. distilled water. One per cent acetic acid was added until a spot test with methyl red matched a spot standard of pH 5.0 acetate buffer plus methyl red. Heavy flocculation promptly followed, and the mixture was centrifuged. Glass electrode readings on the supernatant were usually between pH 5.2 and 5.5. The precipitate was stirred with 8 ml. veronal-buffered saline and the pH adjusted to 7.4 by cautious addition of 0.1 N NaOH. Prothrombokinase solutions were kept in the refrigerator when not in use, but were usually employed on the day of preparation. Several variations of this procedure were tried, both simpler and more elaborate, but most of the experiments were performed on this type of preparation. Prolonged centrifugation after the second adsorption with $BaSO_4$ made little difference in the behavior of the product.

Prothrombin.—According to recent reports prothrombin preparations of very high potency develop thrombin when incubated with calcium (Ferguson, Travis, and Gerheim, 1947) and may also show slight conversion in physiological saline (Ware, Guest, and Seegers, 1947). Therefore, mere high potency carries no guarantee that the prothrombin preparation is free of activating influences. For the present study, minimum contamination with activators was more important than high specific activity; and the work was facilitated by an abbreviated method for preparing a dilute prothrombin reagent which was stable in the presence of calcium ions.

The method was derived from that of Seegers, Loomis, and Vandenbelt (1945), modified and abbreviated as follows:—

136 gm. frozen euglobulin was dispersed in 250 ml. distilled water with a Waring blendor. To this was added 250 ml. cold oxalated salt solution (1.8 per cent NaCl: 1 per cent $K_2C_2O_4 \cdot H_2O$) and enough 0.1N NaOH to bring the pH to 7.4. Undissolved material was removed by centrifugation and the pH was readjusted to 7.4, if necessary. The solution was transferred to a one liter Erlenmeyer flask and kept in a 51°C. water bath for 30 minutes with frequent agitation of the flask and its contents. The flask was then put in cold tap water for a few minutes and then allowed to stand for $\frac{1}{2}$ hour at room temperature or several hours in the refrigerator. After this, the heat-coagulated fibrinogen was removed by centrifugation.

The heated globulin was stored in the refrigerator for 2 days or longer, during which it became less rapidly activatable by calcium. Any additional sediment was removed by centrifugation; and 100 ml. of globulin solution was treated with 28 ml. $Mg(OH)_2$ suspension prepared by the method of Seegers *et al.* (1945). After 15 minutes at room temperature the $Mg(OH)_2$ was collected by centrifugation in lusteroid tubes, and then mixed with 100 ml. distilled water at room temperature. The mixture was thoroughly stirred for 15 minutes, with particular care to break up all clumps, and then centrifuged. After three such washes, the $Mg(OH)_2$ sediment was stirred thoroughly with 100 ml. acetate mixture. Any material not dissolved in 1 hour was centrifuged out. The solution was dialyzed in a rocking dialyzer for $1\frac{1}{2}$ hours, during which 18 liters of 0.9 per cent NaCl were run through the dialyzer. The solution was then dialyzed in the refrigerator against several changes of veronal-buffered saline, over a period of 24 hours. At this time, the prothrombin solution was at pH 7.4, and after centrifuging to remove a slight amount of precipitate, the solution was stored in the freezer.

Lipoid Thromboplastin.—That fraction of bovine brain which was soluble in ether, but not in acetone.

To prepare a stock thromboplastin suspension, 300 mg. of the solid was triturated with 3 ml. veronal-buffered saline. This suspension, when diluted to 10^{-4} in a prothrombin activation mixture, still accelerated the appearance of thrombin. It lost very little of its activity when heated at 60°C. for 10 minutes.

Determination of Coagulation Time.—In an earlier paper (Milstone, 1942), a method was described for assaying thrombin preparations of high potency, using a standard thrombin preserved in glycerin. In the present work, the demands of the situation were different. Here, it was of chief importance to determine how much of a given prothrombin solution had been converted to thrombin at any given moment; in other words, to estimate thrombin in the presence of prothrombin at a sharply defined time, and further to make such estimates at several consecutive intervals. Therefore, in this study, certain operations were avoided, which although promising greater volumetric accuracy would have made timing less precise or consecutive repetition more difficult.

0.1 ml. of the solution to be assayed was carefully measured in the last 0.1 ml. of a 1.0 ml. serological pipette. With a finger over the top of it, the pipette was introduced into a 10 × 75 mm. pyrex tube containing 0.3 ml. oxalated fibrinogen and poised with its tip a few millimeters above the level of the fibrinogen. At the moment when a running stop-watch passed the zero mark, the thrombin was blown into the fibrinogen with sufficient force to cause rapid mixing. (The effectiveness of this procedure was checked by blowing 0.1 ml. of a dye solution into 0.3 ml. fibrinogen.) The blowing was rapidly repeated 2 or 3 times and mixing was promptly continued for another 5 seconds by rapid oscillation of the tube. The coagulation process was followed by repeatedly tilting the tube; and the coagulation time was taken as the interval between the addition of thrombin (zero on the stop-watch) and the instant when the clot would hold its position with the tube inverted. Coagulation times longer than 25 minutes tended to be erratic.

Estimation of Thrombin in the Presence of Prothrombin

With the present materials and methods, it was found that the speed of coagulation was directly proportional to the concentration of added thrombin, within the limits of experimental error. To verify this relationship, a thrombin solution was diluted with the routine buffer-calcium medium, and the coagulation times were determined when each of the dilutions was tested with oxalated fibrinogen. The results are represented by the open circles in Fig. 1, where the reciprocal of the coagulation time, *i.e.* the speed of coagulation, is plotted against the known relative concentration of added thrombin. These data, together with others, showed that deviations from the straight-line function were haphazard and not greater than would have been expected from the experimental error, an appreciable share of which was due to variations in pipettes. Hence, it was at least as accurate to use the simple straight-line relationship as it would have been to use any one empirical curve obtained at a particular time.

Although any number could have been used to convert coagulation time to relative speed of coagulation, 3,000 was chosen because it was convenient to have a coagulation time of 30 seconds represent a thrombin concentration of "100," or, in algebraic form, Relative thrombin concentration $= \dfrac{3{,}000}{\text{C.T.}}$. On this basis a solution which clotted fibrinogen in 60 seconds would be said to have a relative thrombin concentration of "50," etc. Reducing the present results to terms of some arbitrary standard thrombin would not have materially increased the significance of the principles demonstrated, and therefore the assay
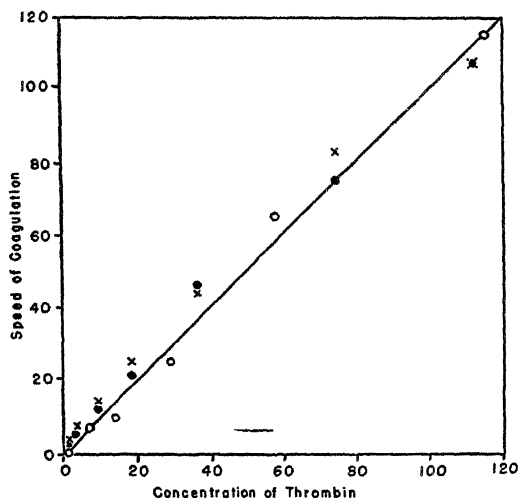
FIG. 1. Relation between speed of coagulation and concentration of thrombin. Open circles, dilutions of thrombin in buffer—0.0025 M Ca. X's, rapid dilutions of thrombin in prothrombin—0.0025 M Ca. Solid circles, rapid dilutions of thrombin in buffer—0.0025 M Ca. 0.1 ml. thrombin dilution plus 0.3 ml. oxalated fibrinogen. Speed of coagulation = 3,000/coagulation time.

values are not meant to bear any definite relationship to various thrombin units which have been employed. However, they will be used in any given experiment, as if they had the connotation "100 units per ml. thrombin solution," etc.

It was further shown that the thrombin assay values were not appreciably affected by the presence of prothrombin. For example, a mixture of 20 per cent thrombin and 80 per cent prothrombin gave in the usual test a coagulation time about the same as that given by a mixture of 20 per cent thrombin and 80 per cent buffer-calcium medium. From this it might be expected that at the moment when a full strength prothrombin had become 20 per cent activated, it, too, would give the same coagulation time, reflecting the fact that 20 per cent of it was thrombin at the time of sampling. In the main this is correct,

with a minor complication which is partly taken into account in the following test, and need not be discussed until later.

The experiment was performed on crude preparations, so that a certain amount of activator material would be brought into the mixture by both the prothrombin and the thrombin. Thrombin was diluted with prothrombin in such a manner that the total concentration of thrombin plus prothrombin was always the same and the concentration of calcium was maintained at 0.0025 M in all cases. These manipulations entailed the admixture of prothrombin, calcium, and activator material, and were therefore performed rapidly; and each mixture was assayed for thrombin immediately. The results are portrayed by the x's in Fig. 1, and are to be compared with similarly rapid dilu-
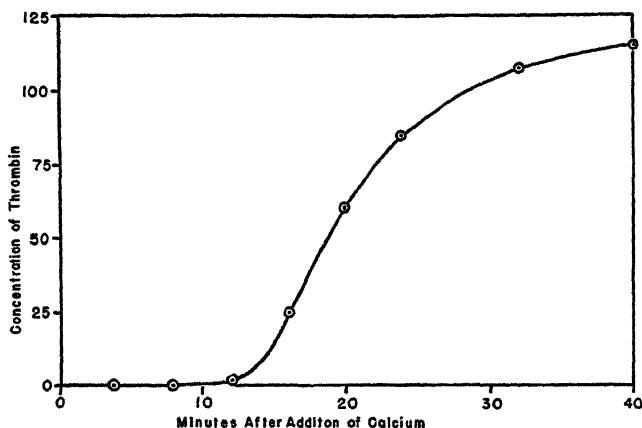
FIG. 2. Activation of crude prothrombin. 0.1 ml. heated globulin and 0.9 ml. veronal-buffered saline, plus 0.1 ml. Ca 0.0275 M.

tions of thrombin in buffer-calcium medium, represented by solid circles. It can be seen that, despite the disadvantages taken, the presence of prothrombin, plus the amount of activator material included, did not appreciably alter the values of the thrombin assays.

The Latent Period in the Activation of Crude Prothrombin

After fibrinogen has been removed from a crude euglobulin solution by heat coagulation at 51°C., the remaining solution still contains everything necessary to produce thrombin upon the addition of calcium. Fig. 2 illustrates the commonly observed pattern of activation. Calcium was added to the diluted globulin, and at the intervals indicated, samples were assayed for thrombin. In this experiment, as in all others where samples were transferred at specified times, the time of sampling was not that at which the sample was removed from the first tube, but the moment when it was added to the next reagent. As shown by Fig. 2, no thrombin was found in the samples taken at 4 and 8

minutes, but a small amount was detectable at 12 minutes. Then, suddenly, thrombin developed with increasing velocity and levelled off toward a plateau value. Activation curves for different crude globulin preparations were found to vary a great deal, especially in the duration of the latent period. However, the general pattern was consistent, and proved to be independent of the presence or absence of fibrinogen or of the manner in which fibrinogen was removed. Similar curves were obtained when the prothrombin was activated in the presence of sufficient fibrinogen to form a solid clot, and also when the crude globulin had been previously defibrinated by the addition of a minimal quantity of oxalated thrombin. However, it was obvious that the activation pattern depended on some factor in addition to prothrombin and calcium. In a previous study (Milstone, 1942) it was noted that it became more difficult to activate prothrombin by the mere addition of calcium as the prothrombin was purified. At that time, it was found (unpublished data) that the condition for normal activation could be restored by adding a small amount of a by-product fraction; and it was tentatively assumed that the additional factor was the precursor of thrombokinase. As recounted above, such a factor had previously been recognized by others. In conformity with what appears to be the oldest and most widely prevalent usage, this factor will now be called prothrombokinase, bearing in mind that the preparations herein studied may contain an activator complex with more than one significant component.

If now two solutions were available, one containing prothrombin, and the other containing prothrombokinase, a combination of these solutions might duplicate the activation pattern depicted by Fig. 2. If so, it should be possible to inquire what was going on during the latent period. If each factor were separately incubated with calcium, it could be determined whether the preliminary interval was occupied by a reaction involving calcium and one or the other of the two thrombin-producing factors.

Adsorption techniques proved useful in separating the two factors, since prothrombokinase appeared to be less avidly adsorbed by such substances as magnesium hydroxide or barium sulfate than was prothrombin. It was possible to remove all but a trace of prothrombokinase from prothrombin by thoroughly washing the magnesium hydroxide on which the prothrombin had been adsorbed. Deterioration of prothrombokinase during the procedure may well have contributed to the success of the preparation. Starting with a crude euglobulin solution, prothrombokinase was freed of most of the accompanying prothrombin by two adsorptions with barium sulfate. When calcium was added to an appropriate mixture of the prothrombokinase and prothrombin reagents, the activation curve was much like that for crude globulin. This suggested that the effect of the procedures had been essentially that of separation, and that otherwise nothing significant had been introduced or lost.

When the prothrombin reagent was incubated with calcium first, and then

prothrombokinase added, the latent period was sometimes a little shorter than that of the control, but it was always clearly in evidence. In contrast, it was possible to abolish the latent period completely by pre-incubation of prothrombokinase with calcium. From this, it appeared that the latent period was concerned with the activation of prothrombokinase under the influence of calcium.

Further examination of the slight effect obtained by pre-incubation of prothrombin with calcium showed that, as a batch of such material stood at room temperature, samples taken from it at intervals first gave slightly shorter latent periods upon activation with prothrombokinase. As incubation of the prothrombin-calcium mixture continued, however, samples taken from it gave slightly longer latent periods. These findings were taken to indicate that a slight amount of prothrombokinase still remained in the prothrombin reagent. This contaminant became active in the presence of calcium and contributed to a slight shortening of the latent period. But, as incubation was continued, the active trace of thrombokinase deteriorated and the latent periods then became longer. All the results which follow are in harmony with this interpretation.

Kinetics of Prothrombin Activation

Not only did prolonged incubation of the prothrombin reagent with calcium fail to abolish the latent period; it also failed to result in a significant production of thrombin. Consequently, it became convenient to use a mixture of prothrombin and calcium as a single reagent. Often such prothrombin-calcium reagents were stored for several days in the refrigerator, during which they developed no significant amount of thrombin (no coagulation in 1 hour) and continued to function satisfactorily as prothrombin.

A steady prothrombin-calcium reagent could be used to define the changing status of a prothrombokinase-calcium mixture, because the latter would be responsible for any change in the kinetics of thrombin production.

Calcium was added to prothrombokinase, and at intervals, 0.1 ml. samples were transferred to 0.9 ml. samples of prothrombin-calcium reagent, the concentration of calcium being 0.0025 M throughout. Then, in each case, a prothrombin activation curve was obtained as already described for Fig. 2. Preliminary experiments at room temperature showed that the activation of prothrombokinase was too rapid to permit such detailed study and that subsequent deterioration was likewise rapid. Therefore, the activation of prothrombokinase was performed in a water bath kept in the refrigerator. Further orienting experiments revealed that when the activation of prothrombin was also performed in the cold, these latter reactions were very slow and the procedure was cumbersome. Therefore, prothrombokinase was activated in the cold, whereas both the activation of prothrombin and the assay of thrombin

by coagulation of fibrinogen were carried out at room temperature. It will be noted at this point that we are already dealing with a three-stage procedure, in which the experimental steps correspond to the three theoretical stages: (1) activation of prothrombokinase; (2) activation of prothrombin; (3) coagulation of fibrinogen (Milstone, 1947).

Some of the results are presented in Fig. 3, which focuses attention on the early portions of the prothrombin activation curves. In this experiment, the
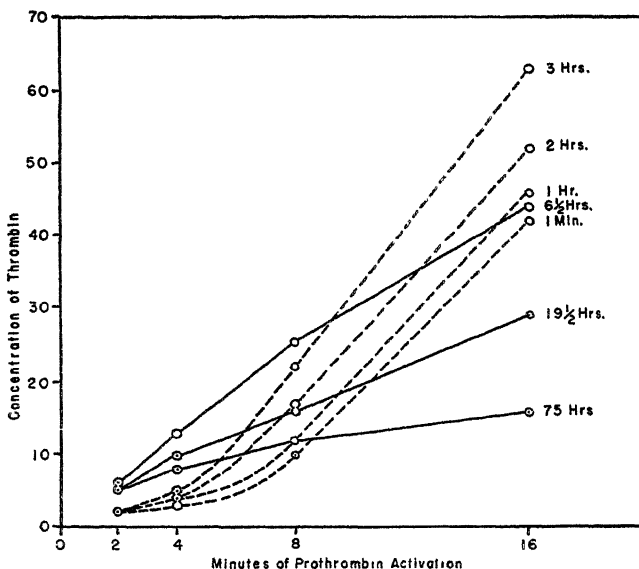
FIG. 3. Successive prothrombin activation curves obtained with samples of prothrombokinase in progressive stages of activation (broken lines), and decline (solid lines). 5.0 ml. cold prothrombokinase plus 0.5 ml. Ca 0.0275 M. Kept cold. 20 ml. prothrombin plus 2 ml. Ca 0.0275 M. At room temperature. The time at the right end of each curve is the age of the prothrombokinase-calcium mixture when 0.1 ml. of it was transferred to 0.9 ml. prothrombin-calcium mixture.

latent period was unusually short at the outset, so that its subsequent obliteration was not as spectacular as in some cases. Nevertheless, the data collected during the run offer a detailed description of the manner in which the latent period is progressively shortened and finally abolished.

During the first 3 hours of prothrombokinase activation, the curves for activation of prothrombin showed that the former was steadily gaining in capacity to activate prothrombin rapidly. In addition, all the early curves, portrayed by broken lines, had a sharp upsweep, indicating that the rate of thrombin production was being accelerated during the course of the reaction. In these curves, furthermore, the *initial rate* of prothrombin activation was close to

zero at first, and then slowly increased as prothrombokinase was activated for 3 hours. In the prothrombin activation curve obtained at $6\frac{1}{2}$ hours, the circumstances were quite different. There, activation of prothrombin began with maximal velocity; and the rate of activation was never accelerated thereafter, but rather it steadily diminished. Despite the fact that the $6\frac{1}{2}$ hour curve started out so much faster than the earlier curves, it soon fell below them. This suggested that, while the prothrombokinase had been converted to the active form, some activity had been lost through deterioration or side reactions, so that the sum of active plus potential thrombokinase was less than when the prothrombokinase-calcium mixture was fresher. That the thrombokinase was actually deteriorating was demonstrated by the curves obtained at $19\frac{1}{2}$ hours and 75 hours.

In view of the possibly enzymatic nature of thrombokinase, it was of particular interest to inquire how closely the prothrombin activation curves approached the theoretical curves for a unimolecular reaction. In such a case, the function $\log \dfrac{A}{A - a}$ should give a straight line when plotted against activation time t, where A equals the maximum thrombin value at the plateau of the activation curve and a represents the amount of thrombin at time t. In such fashion, data of Fig. 3 plus some additional data collected during the experiment are replotted in Fig. 4. There it is seen that curves obtained with partially activated prothrombokinase turn upwards, whereas those curves depicting the activation of prothrombin by ripe thrombokinase closely approximate the straight lines characteristic of unimolecular reactions.

This experiment also gives further evidence that thrombin does not directly catalyze the activation of prothrombin. If it did, all the curves in Fig. 3 would be expected to turn upwards, and none of the curves in Fig. 4 should so closely approximate the unimolecular form.

Estimation of Thrombokinase

As just demonstrated, the activation of prothrombokinase can be followed by comparing the prothrombin activation curves resulting from serial tests. It could have been followed more simply, in the experiment of Fig. 3, by determining how much prothrombin was activated in the first 4 minutes in each test. As is evident from Fig. 3, the activation at the 4 minute intercepts of the curves became progressively greater as the prothrombokinase was incubated with calcium for $6\frac{1}{2}$ hours. In the later work, more concentrated solutions of prothrombokinase were used, with the result that 2 minute intercepts were more appropriate; otherwise the technique was the same. While this device furnished a means of following the activation of prothrombokinase, it was further desirable to reduce the data to a quantitative basis.

To accomplish this, a solution of prothrombokinase was mixed with calcium
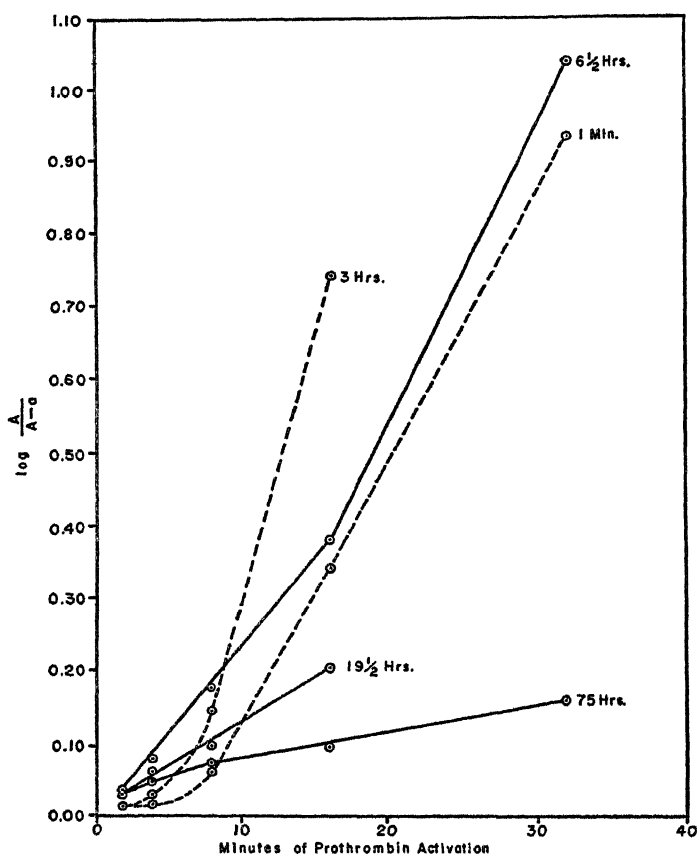
FIG. 4. Activation of prothrombin plotted as a unimolecular reaction.   Same experiment as Fig. 3.
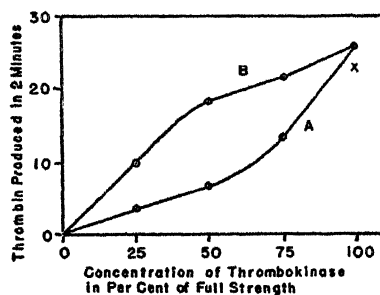
FIG. 5. Activation of prothrombin with increasing concentrations of thrombokinase.   A, dilutions of thrombokinase in buffer—0.0025 M Ca.   B, dilutions of thrombokinase in prothrombokinase—0.0025 M Ca.

in the cold, and the course of activation followed by the above method. When the activity had fully developed, the thrombokinase was promptly diluted with buffer-calcium solution. For each dilution, it was determined experimentally how much prothrombin would be activated by it in 2 minutes. The results are plotted in Fig. 5, and demonstrate that the concentration of added thrombokinase determined the initial rate of prothrombin activation. This would be expected if thrombokinase were an enzyme. However, the empirical relationship is sufficient basis for the quantitative estimation of thrombokinase.

As previously mentioned, the initial rate of prothrombin activation is practically nil when prothrombokinase is used, in place of thrombokinase. This would offer a simple way of estimating the active form in the presence of the precursor, for any initial activation would be due entirely to thrombokinase. The main difficulty is that the activation of prothrombokinase continues, even after its tenfold dilution in prothrombin, no doubt aided by the change from refrigerator to room temperature. Merely running the prothrombin activation in the cold would not solve this problem, because a much longer time would be required for the production of measurable thrombin, and this in turn would allow more time for additional activation of prothrombokinase. Perhaps this will be less troublesome at a later date, when it becomes convenient to use a more concentrated prothrombin "substrate."

It was considered that the activation of prothrombin might be much less sensitive to a decrease in calcium concentration than was the activation of prothrombokinase. If the prothrombokinase-calcium mixture were added to nine volumes of a prothrombin solution that contained no added calcium, then the calcium would be diluted along with the prothrombokinase; and perhaps the activation of prothrombokinase would stop, while the production of thrombin proceeded. But, it was found that the concentration of calcium was important for both reactions. Activation of prothrombin was very slow with 0.00025 M calcium. This conclusion was verified by restoring the calcium to the usual concentration just before the thrombin assays, thus eliminating the possibility that the apparent retardation of thrombin formation was due merely to an effect on the measurement of thrombin.

For the present, it seemed best to make an allowance for the production of extra thrombokinase, in lieu of preventing it. Cold thrombokinase was diluted with cold prothrombokinase in such a way that the total concentration of the two was constant, and calcium was 0.0025 M throughout. The initial rate of prothrombin activation was measured for each dilution, and the results plotted as curve B in Fig. 5. The higher course of curve B, as compared with curve A is largely attributable to the appearance of additional thrombokinase during the 2 minutes of prothrombin activation.

The assumption was then made that an artificial mixture of half thrombokinase and half prothrombokinase would be equivalent to a solution of prothrom-

bokinase that had become half-activated, and similarly for other proportions of active to precursor form.   This seemed plausible, provided the standardization was made anew for each set of materials at the time they were being studied.   With the exception of this assumption, the standardization was empirical, and relative to the peak activity shown by the particular thrombokinase.

As a result of their empirical nature, the standard curves controlled another possible source of error.   Quick (1940) and Owren (1947) have described conditions under which production of thrombin seems to continue for a brief time after oxalation.   In the experiment of Fig. 1, such did not occur to a significant extent.   There the amount of activator material was about twice that present in the experiments of Figs. 2, 3, and 4, but only about half that present in later experiments.   If, with higher concentrations of thrombokinase, this complication did reach a significant level, it was controlled by the empirical curves.   In using them, the over-all effect of the unknown was compared to that of the standard under the same conditions.

Now that it was feasible to estimate thrombokinase in the presence of its precursor, the activation of prothrombokinase could be followed in a quantitative manner.

Kinetics of Prothrombokinase Activation

Table I presents the details of a three-stage procedure, as used in studying the activation of prothrombokinase.   The first stage was represented by a prothrombokinase-calcium mixture which was kept in a water bath at 6°C. Both the prothrombokinase and the calcium solution used to activate it were cold at the start, likewise all glassware that came in contact with these solutions had been prechilled by storage in closed containers in the refrigerator. The second stage was represented by a series of tubes containing the prothrombin-calcium reagent, and the third stage by a series of tubes containing oxalated fibrinogen.   Although these latter materials were also refrigerated during the $7\frac{1}{2}$ hour experiment, they were warmed to room temperature before use in the test.

The gross effects to be observed in such a procedure were so striking that it was an easy matter to follow the reaction while it was in progress, by mere inspection of the raw data.   These are listed in the first and third columns of Table I.   There it can be seen that 2 minutes after calcium was added to prothrombokinase, a sample of this mixture was incubated with prothrombin for 2 minutes, with the result that not enough thrombin was formed to clot fibrinogen in 3600 seconds.   At 120 minutes, the prothrombokinase-calcium mixture had ostensibly changed so little that a similar series of operations resulted finally in a 3,000-second coagulation time.   But at 150 minutes, the change in the prothrombokinase suddenly became evident.   It was now sufficiently active

so that in 2 minutes with prothrombin, it brought forth enough thrombin to produce a clot in 465 seconds. Similar tests at 180 and 210 minutes gave evidence that the prothrombokinase was approaching full activation, as anticipated from previous experience with this type of system. Therefore, when some further increase in activity was recorded at 240 minutes, it was assumed that thrombokinase activity was near its peak. This was the time to set up standard mixtures for the estimation of relative concentration of thrombokinase.

TABLE I

Data Obtained with the Three-Stage Procedure

Tube 1 Activation of pro- thrombokinase	Second series of tubes Activation of prothrombin	Third series of tubes Coagulation of fibrinogen	Tube 1
0.1 ml. of 0.0275 M Ca added to 1.0 ml. prothrombokinase	At time t 0.1 ml. transferred from tube 1 to 0.9 ml. of prothrombin containing 0.0025 M Ca	After 2 min. activation 0.1 ml. transferred from second series tube to 0.3 ml. oxalated fibrinogen	Relative concentra- tion of throm- bokinase at time t
Min. after addition of Ca, t	Thrombin produced in 2 min. 3,000/C.T.	Coagulation time	Per cent of full strength
		sec.	
2	<0.9	>3600	0
30	1.3	2400	3
60	<0.9	>3600	0
90	<0.9	>3600	0
120	1.0	3000	3
150	6.5	465	17
180	20.0	150	62
210	20.0	150	62
240	26.1	115	100
330	23.1	130	95
450	0.1 ml. transferred from tube 1 to 0.9 ml. buffer-calcium	1320	

The activation of prothrombokinase was carried out at 6°C.; the other two reactions were carried out at room temperature, 29°C.

The mixtures were prepared according to the considerations previously discussed, and the technical details are given in Table II. It will be noticed that the entire process of preparing a standard mixture in the cold required 80 seconds. The first 40 seconds, during which prothrombokinase was in contact with calcium were insignificant, because activation starts off so slowly at 6°C. Even the ensuing 40-second contact of thrombokinase with prothrombokinase could have made little difference, again because such mixtures change so slowly in the cold. The results of Table II were actually those which have been presented in Fig. 5.

After these standard mixtures were tested, another test was performed on the original prothrombokinase-calcium mixture which was now 330 minutes old. As shown in Table I, and also by the × in Fig. 5, a slight deterioration had occurred. In a further control, a sample of the thrombokinase was added to buffer-calcium solution instead of prothrombin, and 2 minutes later the mixture was assayed for thrombin, giving a clot in 1320 seconds. This indicated that

TABLE II

Standard Mixtures

Relating Observed Rate of Prothrombin Activation to:
A. Relative Concentration of Added Thrombokinase
B. Per Cent of Thrombokinase in a Thrombokinase-Prothrombokinase Mixture

Tube 1	Tube 1		Tube 3	Tube 2
Relative concentration of thrombokinase	Thrombokinase at peak activity*	Buffer-Ca mixture	Coagulation time	Thrombin produced in 2 min.
	ml.	*ml.*	*sec.*	
A 25	0.25	0.75	840	3.6
50	0.50	0.50	440	6.8
75	0.75	0.25	220	13.6
100	Undiluted		115	26.1
		Fresh‡ prothrombokinase-Ca mixture *ml.*		
B 25	0.25	0.75	300	10
50	0.50	0.50	165	18.2
75	0.75	0.25	140	21.4
100	Undiluted		115	26.1

* Peak activity—240 to 330 minutes after Ca added—*cf.* Table I.

‡ These mixtures were each 40 seconds old at the time they were added to the thrombokinase. A fresh prothrombokinase-Ca mixture was prepared for each thrombokinase-prothrombokinase mixture; and all were prepared cold. The thrombokinase-prothrombokinase mixtures were 40 seconds old at the time a 0.1 ml. sample was added to 0.9 ml. prothrombin containing 0.0025 M Ca. Otherwise the general procedure was the same as that outlined in Table I, and the same materials were used.

even 2 adsorptions with barium sulfate had not removed all the prothrombin from the prothrombokinase reagent, and that the residual prothrombin became active along with the prothrombokinase. On a quantitative basis, the resulting error in the estimation of thrombokinase was not great, not only because of the small difference involved, but also because use of the standard thrombokinase-prothrombokinase mixtures would be expected to allow for this factor.

Another control, performed 6 hours after the beginning of the experiment showed that the prothrombin-calcium reagent still behaved quite the same as it

had at the beginning. When a fresh prothrombokinase-calcium mixture was tested with it, not only did a zero value for initial rate of activation result, but, further, the activation curve through the first 16 minutes was practically the same as that which had been obtained with the original prothrombokinase-calcium mixture at the start. Although this behavior was usually approached by the prothrombin-calcium reagents used in these later experiments, it may be stated that other types of prothrombin preparations have been found to give highly variable activation curves as they aged, even though they did not produce detectable thrombin when incubated with calcium. Both evidences of stability are required in order that the prothrombin be dependable in a three-stage procedure.

The coagulation times listed in the third column of Table I were converted to values for thrombin, as listed in the second column. From the amount of thrombin produced in 2 minutes, values for relative concentration of thrombokinase were derived by use of Fig. 5. These, listed in the last column of Table I, give a quantitative expression for what has already been observed in a general way—that the activation of prothrombokinase proceeded imperceptibly for the first 2 hours, rapidly increased during the 3rd hour, and leveled off at the end of the 4th. The data are plotted in Fig. 6 where the circles represent experimental points and the curve is the theoretical for an autocatalytic reaction. From one of the calculated parameters presented with Fig. 6, it may be concluded that a fair approximation would result if the entire process were treated as a simple autocatalytic reaction in which 0.006 per cent of the total potential activity was in the active form at the start. That the reaction actually is complicated by deterioration and possibly by side reactions has been mentioned above.

Further evidence that the activation of prothrombokinase involves an autocatalytic or chain reaction soon appeared. A fresh mixture of prothrombokinase and calcium was seeded with an additional 5 per cent of fresh thrombokinase. While the seeding increased the initial percentage of active form, it did not affect the total concentration of thrombokinase plus prothrombokinase, or the concentration of calcium. Followed in the cold, the activation of the seeded prothrombokinase reached its peak at the time the curve for the unseeded control was just beginning to leave the base line.

Activation of Prothrombokinase at Room Temperature

The activation of prothrombokinase can also be studied at room temperature. For exploratory work, this procedure is rapid and convenient. Here, the use of thrombokinase-prothrombokinase standards is not feasible, because at room temperature such mixtures change too fast in relation to the time required to make the necessary manipulations. Nevertheless, an arbitrary conversion chart can be made by employing a straight line instead of curves like those of

Fig. 5. The effect of this substitution is to distort the shape of the curves, and to shift them only slightly along the time axis. This was verified by replotting the data of Fig. 6, using a straight line to convert observed rate of prothrombin activation to concentration of thrombokinase.

Whereas such an approximation was useful in enabling a graphic representation of results, the general effects to be described were large and clearly dis-
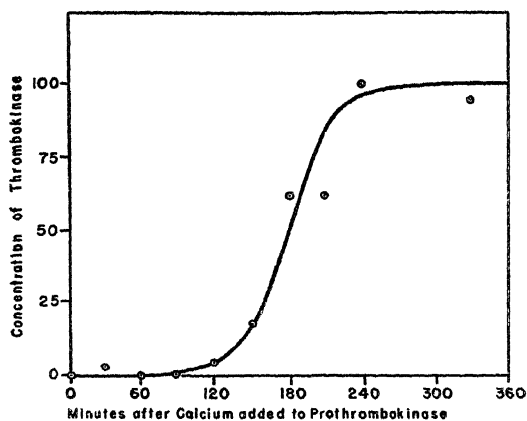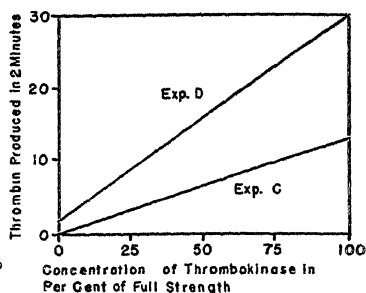
FIG. 6   FIG. 7

FIG. 6. Activation of prothrombokinase at 6°C. Circles, experimental points. Smooth curve, calculated from equation for simple autocatalytic reaction:

$$KAt = 2.3 \log \frac{A(A_e A_0)}{A_0(A_e A)} \qquad \text{(Kunitz and Northrop, 1936)}$$

where

$$K = 0.000543$$
$$A_e = 100 \text{ per cent of peak activity}$$
$$A_0 = 0.006 \text{ per cent of peak activity}$$
$$A = \text{per cent of peak activity at time } t$$

FIG. 7. Arbitrary chart for converting initial rate of thrombin formation to relative concentration of thrombokinase.

cernible from the raw data. Table III, which partitions the observed results from the calculated data, illustrates this point. In Experiments C and D, large differences in results obtained, bear witness to the fact that seeding accelerated the activation of prothrombokinase.

Experiment D is also of interest because it presents an example of another complication occasionally encountered. Here the initial rate of thrombin production was measurably above zero, no matter how fresh the mixture of prothrombokinase and calcium. This may have been due to the contamination of the prothrombin-calcium reagent with a trace of thrombokinase, larger than usual. The complication was treated as a blank when the arbitrary lines were

drawn for converting initial rate of prothrombin activation to concentration of thrombokinase. The largest blanks encountered in any three-stage experiments were those of Experiment D of Table III, and the early experiment presented in Fig. 3.

After the coagulation times of Table III had been converted to values for initial rate of thrombin production, an arbitrary graph was drawn for each experiment, as illustrated in Fig. 7. For Experiment C the line was drawn from

TABLE III

Activation of Prothrombokinase at Room Temperature, Followed by Three-Stage Procedure. Acceleration by Seeding with One-Eleventh Volume of Thrombokinase

Observed data					Calculated data							
Tube 1	Third series tubes				Second series tubes				Tube 1			
Minutes after Ca added to prothrombokinase, t	Coagulation time, sec.				Amount of thrombin produced in 2 min., 3,000/C.T.				Relative concentration of thrombokinase at time t			
	Exp. C Control	Exp. C Seeded	Exp. D Control	Exp. D Seeded	Exp. C Control	Exp. C Seeded	Exp. D Control	Exp. D Seeded	Exp. C Control	Exp. C Seeded	Exp. D Control	Exp. D Seeded
1	>3600	1440	1860	570	<0.9	2.1	1.6	5.3	0	18	0	13
2	>3600	570	1800	180	<0.9	5.3	1.7	17	0	42	0	53
5	>3600	270	1620	115	<0.9	11	1.9	26	0	85	1	87
8	1920	270	270	105	1.6	11	11	29	13	85	33	97
12			100				30				100	
16			110				27				90	
20	240		140		13		21		100		68	

Tube 1, Second series tubes, and Third series tubes have the same significance as in Table I, except for the temperature difference.

In seeding the mixtures, the thrombokinase was added to the prothrombokinase 15 to 30 seconds after the calcium was added.

Any sample of prothrombokinase was always seeded with a sample from its own batch, freshly activated.

the blank value of zero to the peak value of 13. For Experiment D, the line was drawn from 1.6 to 30. From this chart were derived the values for relative concentrations of thrombokinase which are entered in Table III. They show, in numerical fashion, that seeding with thrombokinase hastened the activation of prothrombokinase.

The three-stage procedure at room temperature was then used for several exploratory experiments. Fig. 8 presents an example. First a batch of prothrombokinase was activated by the addition of calcium and the conversion process was followed as just described. The development of thrombokinase is plotted in Fig. 8 as "first control." When peak activity was reached, a portion was heated at 60°C. for 10 minutes, then promptly cooled to room temperature.

Eighty-five minutes after the start of the first activation, two parallel tests were run, starting 1 minute apart. One of these was seeded with one-eleventh volume of heated thrombokinase, the other with unheated. Four hours after the start of the first control, a final control was run on unseeded prothrombokinase.

It is evident from Fig. 8 that both thrombokinase and its precursor were labile under the conditions of the experiment. In each test, thrombokinase declined rapidly from its peak value. The activation curve for the final control was different from that for the first control. This indicated that, in 4 hours, a detectable change had occurred in the prothrombokinase, even without the addition of calcium. Indeed, prothrombokinase solutions were regularly so
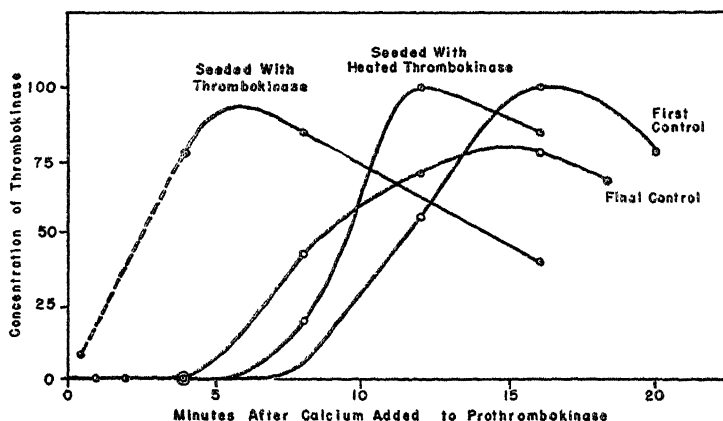
FIG. 8. Three-stage tests comparing heated with unheated thrombokinase in their capacity to accelerate the activation of prothrombokinase. In seeding the mixtures one-eleventh volume of thrombokinase was added 30 seconds after calcium.

unstable that they were used only on the day of preparation, with rare exceptions. It was also necessary, in order to achieve comparable conditions, to run each test at the same time as that to which it was to be compared.

In Fig. 8, it is seen that heated thrombokinase caused a slight, doubtful acceleration, as compared with the definite acceleration effected by the unheated thrombokinase. Additional tests showed that the presence of heated thrombokinase did not prevent an inoculum of the unheated agent from hastening the activation process. Finally, the heated sample was found to cause very slow production of thrombin as compared with an unheated sample of the same age.

When a thrombokinase solution was subjected to one adsorption with barium sulfate, it lost most of its capacity to accelerate the activation of prothrombokinase, and also most of its power to activate prothrombin. Control tests showed that a treated thrombokinase solution did not prevent an untreated

solution from exerting its characteristic effects. Neither were these activities hampered by the addition of a buffer-calcium solution which had been treated with barium sulfate.

DISCUSSION

The blood-clotting process has been partitioned into three primary reactions, carried out in three successive test tubes. The separation, although imperfect, has enabled the development of quantitative techniques for estimating thrombokinase and for following the activation of its precursor. With these new techniques, results have been obtained which may be summarized and interpreted as follows:—

$$1. \text{ Prothrombokinase } \xrightarrow{\text{thrombokinase(?) + Ca}} \text{ thrombokinase}$$

$$2. \text{ Prothrombin } \xrightarrow{\text{thrombokinase + Ca}} \text{ thrombin}$$

$$3. \text{ Fibrinogen } \xrightarrow{\text{thrombin}} \text{ fibrin}$$

where all precursors are substances closely associated with the plasma globulins and all three reactions are enzymatic. The position of Ca over the arrows is meant to imply only that ionic calcium conditions the reactions.

The activation of prothrombin by thrombokinase followed the course of a theoretical unimolecular reaction, moreover the concentration of thrombokinase determined the initial rate of prothrombin activation. This behavior suggests that it is an enzyme. Even so, thrombokinase appears to be distinct from the fibrinolytic enzyme (plasmin) (Milstone, 1947).

If thrombokinase is produced by a simple autocatalytic reaction, then it should possess not only the power to activate prothrombin, but also the capacity to accelerate the activation of prothrombokinase. Solutions of thrombokinase actually showed both properties. Moreover, solutions of its precursor showed neither, indicating that both functions were later acquired during incubation with calcium. That both were lost when thrombokinase solutions were heated at 60°C. or subjected to adsorption with barium sulfate, is also in harmony with the view that both functions belong to a single entity. Furthermore, a fair mathematical approximation resulted when the activation of prothrombokinase was treated as a simple autocatalytic reaction, with a minute amount of active form present at the start.

Despite this suggestive evidence, it has not been proven that thrombokinase, *per se*, accelerates the activation of its precursor; and other, more complicated, interpretations are still under consideration. The thrombokinase solutions were crude and may have contained other significant components, in addition to a small amount of thrombin. The evidence presented corroborates previous reports that thrombin does not directly catalyze the activation of prothrombin;

but its possible effect on the activation of prothrombokinase remains undetermined. However, the present diagram may represent the basic coagulation mechanism, on which a variety of accelerating or retarding influences can impinge.

The present use of the term, thrombokinase, is in accord with earlier usage in the following respects: Morawitz (1904) and Mellanby (1909, 1917) associated thrombokinase with protein fractions. Both likened it to enterokinase, and Mellanby considered it an enzyme. Furthermore, they applied the term alike to blood and tissue factors, basing their concept of thrombokinase(s) primarily on function and properties. Rumpf (1913) emphasized that its action was not identical with that of heat-stable lipoid factors. During the present work, experiments not detailed here have suggested that thrombokinase plus calcium activates prothrombin directly, whereas lipoid thromboplastin plus calcium does not. Lipoid thromboplastin seems to hasten coagulation in some other manner.

Prothrombokinase is a logical name for the precursor, and the most widely recognized. Lenggenhager (1936) wanted to replace it with prothrombokinin, because he felt the latter would not carry enzymatic implications to which he objected. Widenbauer (1943) and Reichel (1944) used prothrombokinase, Astrup (1944) prokinase. Laki (1944) and Quick (1947a) introduced new terms, but indicated that they were equivalent to the older ones. In addition, factor V (Owren, 1947), prothrombin A (Quick, 1947b), labile factor (Quick, 1947b), and other designations have recently been applied to what seems to be the same principle.

The problems involved in charting the production of thrombokinase have been attacked with different techniques by Laki (1943) and Owren (1947), both of whom concluded that the autocatalytic phenomenon was concerned with the production of an activator.

The distinguishing features of the present analysis are that the procedure is carried out in three steps that correspond directly to the three theoretical stages, and that thrombokinase and thrombin are separately estimated, each in terms of its defining property. It is believed that this type of analysis permits more secure interpretations and furnishes the groundwork on which more precise methods can be built.

SUMMARY

1. Blood-clotting mechanism has been analyzed by a procedure which devotes a separate experimental step to each of the three primary reactions:

1. Prothrombokinase → thrombokinase
2. Prothrombin → thrombin
3. Fibrinogen → fibrin

2. Activation of prothrombin by thrombokinase followed the course of a unimolecular reaction, and the concentration of thrombokinase determined the initial rate. By this relation thrombokinase was measured, and the activation of its precursor was charted.

3. When the activation of prothrombokinase was plotted against time, the experimental points fell close to the theoretical curve for a simple autocatalytic reaction. Moreover, the process was accelerated by seeding with a small amount of crude thrombokinase. It was concluded that the activation of prothrombokinase involves an autocatalytic or chain reaction.

4. The three-stage procedure made possible the separate estimation of the power to activate prothrombin, on one hand, and the capacity to accelerate the transformation of prothrombokinase on the other. Drastic losses of both activities occurred when crude thrombokinase solutions were heated at 60°C., or adsorbed with barium sulfate.

5. The concentration of calcium was important for the normal progress of prothrombin activation, and also for the transformation of prothrombokinase.

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VOLUME CHANGES IN HEMOLYTIC SYSTEMS CONTAINING RESORCINOL, TAUROCHOLATE, AND SAPONIN

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Hemolysis by a number of different lysins (saponin, the bile salts, and certain alcohols among others) is preceded by a loss of K from the human red cell (Ponder, 1947 *a*, *b*, *c*), and also, in the case of at least some lysins (alcohols, bacterial toxins, saponin, and the bile salts among others) by an increase in cell volume.

Prolytic volume changes can conceivably occur in two ways. Each cell of the system may start to swell as soon as, or shortly after, the reaction between its components and the lysin begins, and the swelling may continue up to the time at which the cell hemolyzes. The swelling need not necessarily be the same in the case of all cells, since different resistances to the lysin have to be taken into consideration, nor need the point corresponding to maximum swelling and lysis be the same for all cells, since there may be differences in critical volume; as regards the cells of the system as a whole, however, the mean volume V will increase as a continuous function of time from the initial mean volume V_0 to the mean critical volume V_h. A volume increase of this kind could be detected by measuring the volume concentration of the cells, using any suitable method, and by making suitable allowances for the amount of hemolysis present in the system.

Alternatively, the volume increase may be a sudden terminal event, occurring just before the cell hemolyzes. In this case it would be detected only by the measurement of the volume of the individual red cell, and the mean volume of the intact cells of a hemolytic system would not increase with time.[1]

The first of these two kinds of volume change affects the mean volume of the intact red cells of a hemolytic system the most, and the largest effects may be expected to occur in systems containing concentrations of lysin great enough to hemolyze not only the cells of least resistance, but cells of mean resistance and of even greater resistances. The volume of the intact cells in

[1] Photographic measurements of single red cells show that an increase in diameter precedes hemolysis in systems containing saponin and sodium taurocholate, but not in systems containing complement and amboceptor (Ponder, 1923). The errors attached to the calculation of cell volume from photographic measurement are very large; the sequence of shape transformations, moreover, was not understood at the time when these measurements were made. In consequence, the values for the critical volumes obtained in the investigation should have no reliance placed upon them.

such systems can be measured by the modification of the Hamburger (or van Allen) hematocrit method introduced by Guest and Wing (1939, 1942), in which the volume of the intact cells and the percentage of complete hemolysis are measured simultaneously. The measurement of red cell volume by this method takes from 15 to 30 minutes (the time of spinning in a high speed hematocrit), and so the method can be applied conveniently to the type of hemolytic system in which slow cation losses are most easily demonstrated. The simultaneous measurement of volume changes and of K-Na exchanges has a special interest in view of the hypotheses which have been advanced to account for the volume increases on the basis of the permeability to cations.

Methods

The cells of freshly drawn heparinized human blood are washed three times with 172 m. eq./liter NaCl, and suspended in the same medium so as to give a suspension with a volume concentration of 0.4. Two ml. of this suspension is added to each of a series of dry tubes, the contents of which are then cooled to 4°C. Ten ml. of a known concentration of the hemolysin in 172 m. eq./liter NaCl, cooled to 4°C., is added to the cells in the first tube, 10 ml. of another concentration to the cells in the second tube, and so on. One's aim is to select concentrations of lysin ranging from those less than the concentration which gives just commencing hemolysis in 18 hours at 4°C. (or in some other time at some other temperature), up to a concentration which gives from 50 to 80 per cent hemolysis at the end of the same time at the same temperature. The concentrations which fall within this range have to be found by trial. One tube containing 2 ml. of the cell suspension and 10 ml. of 172 m. eq./liter NaCl is included in the series; this serves as the standard system which contains no lysin.

The contents of the tubes are mixed and allowed to stand for 18 hours at 4°C., with occasional mixing by inversion. At the end of this time, 2 ml. of the contents of each tube in the series is transferred to the cups of cooled Hamburger hematocrit tubes.[2] These are spun for 30 minutes at 4000 R.P.M. in a centrifuge cooled to 4°C. The clear and often Hb-stained fluids in the cups are transferred to a series of vials, and the Hb concentration in each is determined colorimetrically. Each Hb concentration is expressed as a fraction of the concentration which corresponds to complete hemolysis in the system under consideration; call this fraction p.

The length of the column of packed cells in each hematocrit tube is measured and corrected for any differences which may have been found in the bore of the capillary. Call this corrected length h, the particular length found for the system containing no lysin being denoted by h_0.

The tubes containing the series of hemolytic systems (from each of which 2 ml. has already been transferred to the cups of the hematocrit tubes) are centrifuged at a moderate speed in the cold centrifuge; the supernatant fluids are transferred to a series

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[2] These tubes are constructed so that the volume of the cup at the upper end (2 to 3 ml.) is about 10 times the volume of the capillary, which is about 80 mm. long and about 2 mm. in bore. It is sealed at the lower end. These tubes, with any specified cup/capillary ratio, are made by E. Machlett and Sons, New York, N. Y.

of vials, and the K content of each is found with the Perkin-Elmer photometer. Each K content, which is equivalent to a K loss from the cells, is expressed as K_p, the fraction of the total K content of the red cells of the system under consideration. The loss which occurs in the standard system of the series, *i.e.* in the system which contains no lysin, is denoted by K_s.

Since the fraction of the cells which have hemolyzed in the system is p, the fraction remaining intact is $(1 - p)$. These intact cells have a total volume proportional to h, so the volume per cell is $V = h/(1 - p)$. The volume per cell in the standard system containing no lysin is V_0, proportional to h_0, so the fractional increase in volume which the average cell undergoes in the hemolytic system is

$$V/V_0 = h/h_0(1 - p) \qquad (1)$$

The loss of K from the cells is K_p, expressed as a fraction of K_0, the initial content of the cells. The loss of K which can be attributed to the action of the lysin, how-

TABLE I

*Hemolysis, K Losses, and Volume Changes in Systems Containing Resorcinol,
All after 18 Hours at 4°C.*

c	h	p	$\dfrac{h}{1-p}$	V/V_0	K_s	K_p	$K_p - K_s - p$	F
M								
0	34	0.0	34.0	1.00	0.07	—	—	—
0.032	36	0.0	36.0	1.06	—	0.19	0.12	0.12
0.048	38	0.0	38.0	1.11	—	0.36	0.29	0.29
0.064	39	0.01	39.4	1.16	—	0.69	0.61	0.62
0.096	52	0.06	55.5	1.63	—	0.83	0.70	0.74
0.112	37	0.44	66.0	1.93	—	0.90	0.39	0.70

ever, is $K_p - K_s$, where K_s is the small loss which occurs in a standard system containing isotonic NaCl only. In a system containing lysin, some of the loss of K may be due to a fraction p of the cells having hemolyzed and lost all of their K, so the remainder $(K_p - K_s - p)$, represents the loss of K, attributable to the action of the lysin, from the cells which remain intact. The fraction of its initial K which the average intact cell loses is accordingly[3]

$$F = (K_p - K_s - p)/(1 - p) \qquad (2)$$

Relation of the Volume Changes, the K Losses, and the Course of Hemolysis

Typical results obtained with resorcinol, sodium taurocholate, and saponin in systems containing human red cells, after 18 hours at 4°C., are shown in

[3] On the alternative hypothesis that some of the cells lose all of their K, instead of all of the cells losing some of their K, F represents the fraction of the intact cells which have become completely permeable to K and have exchanged this ion for the Na of the surrounding medium. It should be borne in mind that no way has been devised up to the present time, of showing that the one alternative is altogether right and the other altogether wrong.

Tables I, II, and III and in Figs. 1, 2, and 3. The dotted curves marked p show the course of hemolysis; they begin at a value of c on the abscissa for which the fraction of complete hemolysis, p, is zero. The curves shown in heavy line represent the mean volume V, expressed with reference to the initial mean volume V_0, which the intact red cells attain in systems containing various amounts of lysin and at the end of 18 hours at 4°C. In order to render them familiar, these curves may be compared with the curve obtained

TABLE II

Hemolysis, K Losses, and Volume Changes in Systems Containing Sodium Taurocholate, All after 18 Hours at 4°C.

c	h	p	$\dfrac{h}{1-p}$	V/V_0	K_s	K_p	$K_p - K_s - p$	F
ml./10 ml.								
0	32	0.0	32.0	1.00	0.06	—	—	—
2	33	0.0	33.0	1.03	—	0.07	0.01	0.01
4	36	0.0	36.0	1.12	—	0.10	0.04	0.04
6	38	0.03	39.2	1.23	—	0.18	0.07	0.07
8	35	0.11	39.2	1.23	—	0.37	0.20	0.22
10	26	0.32	38.3	1.20	—	0.63	0.24	0.35

TABLE III

Hemolysis, K Losses, and Volume Changes in Systems Containing Saponin, All after 18 Hours at 4°C.

c	h	p	$\dfrac{h}{1-p}$	V/V_0	K_s	K_p	$K_p - K_s - p$	F
γ/10 ml.								
0	35	0.0	35.0	1.00	0.07	—	—	—
50	30	0.17	36.2	1.04	—	0.30	0.06	0.07
100	22	0.45	40.0	1.14	—	0.68	0.16	0.29
150	7	0.82	8.6	1.10	—	0.95	0.06	0.33

by plotting the volume which the intact red cell attains at equilibrium in media of decreasing tonicity (insets of Fig. 2).

Each point on such a curve represents a volume V, expressed in terms of the initial volume V_0, which the cell attains as the result of the intake of water at a rate

$$dV/dt = f \cdot (T_1 - T) \tag{3}$$

i.e., at a rate which is a function of the difference between the tonicity in the cell interior and that in the external medium. When $T_1 = T$, $dV/dt = 0$, there is no further volume increase, and if the volume of the external medium of tonicity T is very large, the volume attained by the cell is

$$V/V_0 = RW \cdot \left(\frac{1}{T} - 1\right) + 1 \tag{4}$$
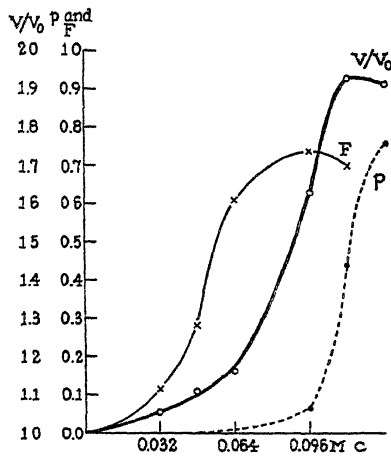
FIG. 1. Hemolysis, K losses, and volume changes in systems containing resorcinol, all after 18 hours at 4°C. Ordinates, volumes V/V_0 of intact cells in terms of their initial volume, fractions of complete hemolysis (p), and fractions of initial K lost by average intact cell (F). Abscissa, concentration of lysin.
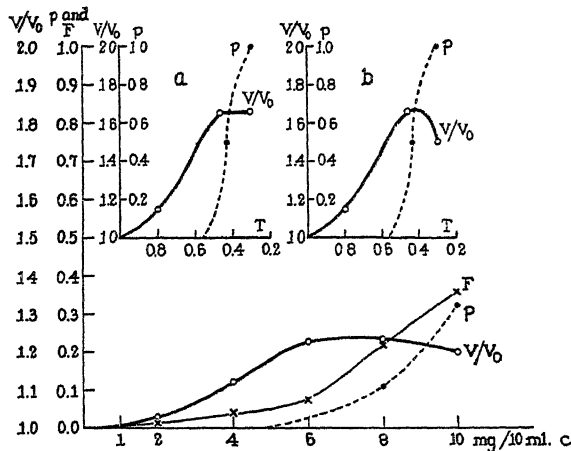
FIG. 2. Same as Fig. 1, but for the lysin sodium taurocholate at pH 6.5. Inset: corresponding curves for a system in which hemolysis is due to hypotonicity. Ordinates as before; abscissae, tonicity in grams of NaCl per 100 ml. For explanation of a and b, see text.

where RW is the fraction of the cell water which is capable of taking part normally in the osmotic process. Strictly speaking, the equilibrium volume is reached only after infinite time, but as a matter of observation it is reached in less than a minute (Ponder and Robinson, 1934). It should be noticed that the relation between V/V_0 and T is

derived on the assumption that the cell is impermeable to cations, osmotic equilibrium being reached by water exchange alone.

If V/V_0 exceeds a certain volume known as the critical volume, the cell hemolyzes. In simple osmotic systems containing hypotonic plasma and human red cells, the critical volume has a value of about 1.6 V_0. This is accordingly the maximum swelling which the intact cells of such an osmotic system can undergo, and in the simplest case a further decrease in T results in each cell reaching the critical volume but in there being progressively fewer intact cells and more hemolysis (dotted curves in the insets of Fig. 2) until lysis is complete. The intact cells which remain after hemolysis begins, however, do not necessarily reach the same maximum value of V/V_0, for as the tonicity is made progressively smaller, the critical volume for hemolysis may become smaller also. This results in the values for V/V_0 reaching a maximum
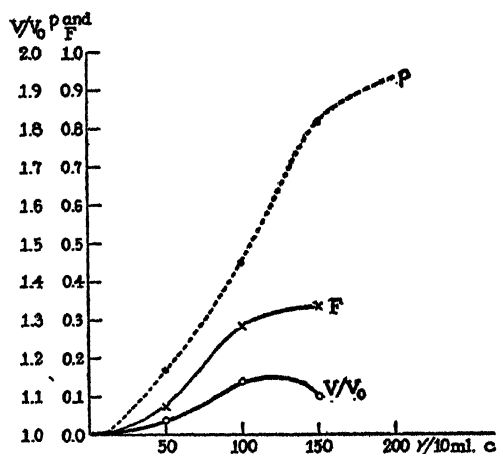
FIG. 3. Same as Fig. 1, but for the lysin saponin at pH 6.5

and then decreasing (inset b of Fig. 2). The decrease cannot continue very far, for a tonicity is soon reached at which even the most resistant cell hemolyzes.

The curves shown in Figs. 1, 2, and 3 are similar to the curves of the insets of Fig. 2 in the way in which they are constructed.[4] They rise, at first convex to the abscissa, to reach a plateau beyond which there is no further swelling of

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[4] In each case the volumes plotted on the ordinates are those obtained after long times in media which produce increasing amounts of hemolysis as the values on the abscissae increase. The difference between the two situations is that there is every reason, both experimental and theoretical, to suppose that equilibrium is reached in the case of hemolysis by hypotonic media, whereas in the case of hemolysis by a lysin the volumes are probably increasing very slowly even after times as long as 18 hours. If they could be shown to be stationary, the "dual hypothesis" for hemolysis would be untenable unless it could be supposed that the cell membrane had become impermeable to cations.

intact cells, but only increasing hemolysis (dotted curves) as the concentration of lysin is increased. This plateau is maintained over a variable range of lysin concentration, but when the concentration is increased still further, the values of V/V_0 tend to fall again; i.e., there is an indication that the critical volume is a function of the lysin concentration.

The curves of Figs. 1, 2, and 3 differ from one another, however, with respect to the maximum value of V/V_0 reached; in systems containing resorcinol (0.112 M), V/V_0 is 1.93, in systems containing sodium taurocholate (6 mg./10 ml. or about 10^{-3} M), V/V_0 is about 1.23, and in systems containing saponin (100γ/10 ml. or about 10^{-5} M), V/V_0 is about 1.14.

While the curves marked V/V_0 in Figs. 1, 2, and 3 show the volumes occupied by the cells of the systems at the end of 18 hours at 4°C., the curves marked F show the fraction of their initial K lost by the intact cells at the end of the same time at the same temperature. These sigmoid curves rise from zero for

TABLE IV

Volume Attained and Fraction of Initial K Lost by Cells in Concentrations of Lysins Barely Sufficient to Initiate Hemolysis

	a_0	V/V_0	F
Resorcinol........................	0.056 M	0.13	0.50
Taurocholate......................	5.0 mg./10 ml.	0.18	0.05
Saponin..........	12.0 γ./10 ml.	0.01	0.01

a system containing no lysin to a maximum value (35 to 75 per cent of the available K per cell) in systems in which there is from 40 to 80 per cent hemolysis. The observation that the maximum values of F are so far short of the total K available shows that some of the cell K is retained in each of the few remaining intact cells, even when there is sufficient lysin present to bring about extensive hemolysis.[5]

It may be helpful to look at the curves in Figs. 1, 2, and 3 in the following way. When just commencing hemolysis is produced after as long a time as 18 hours in a system containing lysin in concentration c, c is substantially the asymptotic concentration for a time-dilution curve with just commencing hemolysis as its end-point. In this concentration, a number of the molecules of the cell structure exist combined with a number of lysin molecules proportional to c; call this number a_0 to indicate that it is great enough to result in the breakdown of the structure of the least resistant cell ($p = > 0$ but < 0.01). The values of c corresponding to a_0 can be read off from the curves which show how p varies with c (dotted in the figures), and the values of V/V_0 and of F can be read off from their respective curves. In this way we get a

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[5] The same kind of result is obtained by applying the same analysis to the curves of Fig. 3 in the paper referred to as Ponder, 1947 a.

correspondence between a_0, a measure of the extent to which the structural components of the cell are combined with the lysin, and V/V_0, the volume which such cells can attain without hemolyzing: we also get a correspondence between V/V_0 and F, the fraction of their initial K lost by the cells when lysis is just commencing. A set of typical results are tabulated in this way in Table IV. Similar tabulations can be made for other values of p, up to the maximum values for which results are shown in the figures.

<div align="center">DISCUSSION</div>

The relation between the curves for the progress of the hemolytic reaction and those for the progressive loss of K from the intact cells is similar to that found in previous investigations (Ponder, 1947 a, b, c). The volume changes alone require discussion.

Since V/V_0 and F are functions of c, the concentration of lysin in the system, it is possible to think of V/V_0 as a function of F, and as related to it as effect is to cause. This is what has been attempted in the "dual mechanism for hemolysis" hypothesis (Davson, 1936, Davson and Danielli, 1938, Davson and Ponder, 1938, 1940), and in the "colloid osmotic hemolysis" hypothesis (Wilbrandt, 1941), the basis of which is that the colloid osmotic pressure of the material in the red cell interior is greater than that in the surrounding medium, that the absence of an osmotic pressure difference is due to the non-diffusibility of cations, and that the swelling of the red cell in systems containing lysin is the result of the cell membrane having become permeable to K and Na: this would terminate a previously existing osmotic equilibrium, and water would move into the cell. The essential features of the dual hypothesis and of the colloid osmotic hemolysis hypothesis have been retained in Jacobs and Stewart's (1947) general treatment of the volume changes resulting from disturbances of ionic equilibrium.

As they now stand, these hypotheses are incomplete in two respects. They say nothing about the conditions under which an osmotic phenomenon, characterized by an entrance of water and an increase in red cell volume, becomes a hemolytic phenomenon, and they tell us nothing about the rate at which the volume changes may be expected to occur. When the hypotheses were first put forward, it was tacitly assumed that the increase in cell volume is limited by the critical volume to which the cell can swell in a hypotonic solution; $i.e.$, that in the case of the human red cell, the critical volume would always be about 1.6 V_0. This does not seem to be so, for V_h is determined by the nature of the lysin in the system, and probably by the lysin concentration also. The fundamental process is accordingly one which determines two things at the same time, (a) the fact that swelling shall occur, and (b) the volume at which the swelling results in a breakdown of the cell. More specifically as regards (b), the number of lysin molecules combined with cell molecules must be able

to determine that V_h is sometimes large and sometimes small. Let us consider these two effects separately.

(*a*) In the model upon the behavior of which the dual hypothesis is based, a permeability to cations, produced by the action of the lysin on the cell membrane, would be followed by a swelling of the cell; under the conditions specified, however, all that we know about the course of this swelling is that it would be infinitely great in infinite time. Given a slow exchange of K for Na, across a membrane permeable to water and in an isotonic system, it can be shown that the rate of the passage of water depends, to a first approximation, on the rate of increase in the concentration of ions in the cell interior. This again depends on the rate of entry of Na and the rate of exit of K, both of which are functions of the driving forces to which these ions are subject and of their mobilities in the membrane. The situation under consideration is such that, in the long run, the cell volume will increase indefinitely, but the initial state can proceed to the final state along so many paths, involving so many variables, that the dual hypothesis cannot even be stated satisfactorily, much less verified.[6] Our observations of volume give values which are attained in finite time, and there is no satisfactory theory with which to compare them; in the meantime, however, it may be noticed that in some systems (*e.g.*, 0.064 M resorcinol) the K loss is very large ($F = 0.6$) while the volume change is quite small (0.16), and that, in systems in which there is no hemolysis, the volume increases are by no means equal for equal K losses produced by different lysins. If these observations do not constitute fatal objections to the dual hypothesis, it is because the hypothesis leaves open so many possibilities as to the behavior of the volume of the red cell in finite times.

(*b*) While a state of cation permeability no doubt results from an effect of the lysin on the cell membrane, and while this would be followed by swelling in the model to which the dual hypothesis refers, there must be a set of superadded conditions which are responsible for the critical volume being large in some systems and small in others. Presumably the reaction of the lysin with the structural components of the cell is again involved, the effect being to decrease the stability of the latter so that various degrees of swelling, small in the case of some lysins and large in the case of others, cause it to break down

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[6] Another way of stating the difficulty is to call attention to the equation for the volume V attained at equilibrium, on the "dual hypothesis" (*i.e.*, the equation corresponding to expression (4)), having $V = \infty$ while at the same time there is no theoretical counterpart to expression (3) in the case of a cell which is exchanging K for Na in an isotonic system. The problem of the ways in which a final state can be reached from an initial state has complexities which have been discussed by Schreinemakers (1938). One can, of course, make a variety of simple assumptions and justify them because they appear reasonable, but this is substituting a qualitative description for the quantitative treatment which is required.

altogether.   There is no objection to thinking of this weakening of the cohesion of the elements of the red cell structure as the result of the same fundamental process as that which brings about the swelling,[7] *i.e.* the reaction between lysin molecules and molecules of the cell structure, but it should be noted that even if the conditions upon which the swelling depends were adequately described, the additional conditions which regulate the upper limit of the swelling would require to be specified also.

SUMMARY

Simultaneous measurement of hemolysis, the volume of the intact cells, and the K lost from the intact cells of systems containing resorcinol, sodium taurocholate, and saponin shows that the volume increases may be conspicuously small while the K losses are large, and that the volume increases are unequal for equal K losses produced by different lysins.   In higher concentrations of the same lysins, the critical volume for hemolysis is a function of the nature of the lysin and of its concentration.

It is impossible to say whether these observations are compatible with the current "dual mechanism" and "colloid osmotic" hypotheses of hemolysis, in which the swelling of the cell is supposed to result from the lysin having made it cation-permeable.   The difficulty to be overcome is that the theory cannot be developed to describe volume changes in finite time unless we know what assumptions to make about the mobilities of K and Na, the forces driving them into and out of the cell, etc.   The experimental results do not suggest, however, that any simple set of assumptions would be satisfactory.

The conditions which regulate the upper limit of the swelling, *i.e.* the point at which a swelling phenomenon becomes a hemolytic phenomenon, are functions of the nature of the lysin and sometimes of its concentration.   They require to be specified by an independent statement, over and above any statement which may be made about the rate at which swelling occurs in the system. The simplest view of the situation is that the conditions which regulate the critical volume and those which regulate the rate of swelling are both functions, as yet undefined, of the reaction which takes place between the lysin and the structural components of the red cell.

[7] The dual hypothesis, with its explanation of swelling as resulting from the cell's becoming cation-permeable, is not the only possible hypothesis by which swelling could be accounted for.   It is at least conceivable that the breakdown of the cell involves an increase in the osmotic pressure of the materials in the cell interior because of a decrease in their molecular association.   This would provide a force which would transfer water and produce swelling; if structural restraints were present, the swelling might even be a limited one at equilibrium.   This possibility is mentioned in order to show that there are models to be considered other than the model upon which the dual hypothesis is based.

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STUDIES IN BIOCHEMICAL GENETICS IN DROSOPHILA

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In recent years, more and more geneticists have been attacking the problem of the mode of gene action. They have used indirect methods, such as subjecting developing organisms to heat, cold, x-rays, ultraviolet rays, or chemicals, to alter development and produce phenocopies and then have attempted to infer the normal action of the gene from these results. Beadle and Tatum and their colleagues have approached the problem more directly by using the biochemical mutants of the bread mold *Neurospora* (1). From these studies they have set up the working hypothesis that each gene regulates a particular step in a particular biosynthesis. In analyzing the effects of genes on development, one basic approach is a determination of the effects of a gene on the metabolic activity of the particular group of cells which will give rise to the altered part. In most animals it is impossible to locate exactly the cells which will form a particular structure, but in *Drosophila* each adult organ develops from a discrete group of cells in the larva called an imaginal disc. In the larva are paired discs (*cf.* Chen (2), or Auerbach (3) for figures) of distinctive sizes, shapes, and positions, which are supported in position in the hemocoele by the tracheal tubes. They can be dissected out of the larva and their oxygen consumptions can be measured by the Cartesian diver ultramicrorespirometer (4, 5). By using these imaginal discs and the Cartesian diver an attack can be made on two basic and interrelated problems of biochemical genetics: How do embryonic tissues in the same animal, which are going to develop into different organs, differ physiologically? And, how do single gene differences produce differences in development?

In an earlier study (6) the Q_{O_2}'s of wild type, "miniature," and "vestigial" wing discs were found to be 20, 18, and 9 mm.^{3} O_2/hr./mg. dry weight tissue respectively. The mutant genes m and vg affecting wing size produce their effects by altering the rate of some chemical reaction in the wing discs of the larva which is reflected by a lowered rate of oxygen consumption. It is interesting to note that the leg discs and probably the other discs as well of the vestigial larva have an unchanged metabolism, or at least have a normal rate

of oxygen consumption. This indicates that genes produce their biochemical as well as their morphological effects only in certain cells of the body, presumably by some interaction of the gene or gene products with specific components of the cytoplasm of those cells. This is supported by Ellenby's finding (7) that the over-all oxygen consumption of vestigial prepupae is the same as that of wild type prepupae.

On the hypothesis that each gene regulates a particular biosynthesis, a search has been made for the enzyme affected by the vestigial gene by adding specific inhibitors or substrates to the buffer solution containing the discs in the diver and measuring the resulting change in the oxygen consumption.

Materials and Methods

The stocks of *Drosophila melanogaster* used were an isogenic wild type established previously (8) and the mutant stocks "vestigial" wings (vg, chromosome 2, locus 67.0), "miniature" wings (m, chromosome 1, locus 36.1), and "four-jointed" legs (fj, chromosome 2, locus 81). Miniature wings are shorter and narrower than usual but have a normal shape and venation. Vestigial wings are reduced to small stumps held outstretched. The legs of four-jointed flies have four instead of five segments in the tarsae. The flies were raised at 23°C. in half-pint culture bottles on corn meal-molasses-agar medium seeded with fresh yeast.

Larvae and prepupae were dissected in a drop of phosphate-Locke solution buffered at pH 7.4 to which was added the substrate or inhibitor being used. The disc and its drop of fluid was transferred to the diver by means of a calibrated braking pipette described by Claff (9). The divers used had a total volume of 7 to 10 mm.³ and "diver constants" (10) varying from 0.78 to 1.44 × 10⁻³. The divers were filled with 1 mm.³ phosphate-Locke plus substrate or inhibitor in the bulb, and 0.5 mm.³ alkali seal and 0.5 mm.³ oil seal in the neck. All respiration determinations were made at a temperature of 27.0°C. and respiration was followed 1 to 2 hours after a 10 minute equilibration period. Each of the figures given in Tables I to V is an average of three to eleven, usually six, separate determinations.

The discs were weighed by the quartz fiber balance described by Lowry (11). The discs were rinsed several times in glass-redistilled water to remove the phosphate-Locke solution, and transferred to a quartz loop in a drop of distilled water. They were dried in an oven at 100°C. for 30 minutes and then weighed. The hooks were then cleaned and reweighed to check the zero point. The balance had been calibrated previously by putting known volumes of standard salt solutions on the hooks, drying, and weighing.

RESULTS

1. Inhibition by Cyanide and Azide.—Since the HCN formed when KCN is put in solution is volatile, the alkali seal used in the divers in experiments with cyanide must contain KCN in the proper concentration to prevent the HCN of the bottom drop from distilling up into the alkali seal. In experiments with 10⁻⁴ M KCN in the bottom drop, the alkali seal mixture was made up of one

part 2 M KCN and one part 0.2 M NaOH; with 10^{-5} M KCN in the bottom drop, a mixture of one part 0.2 M KCN to one part 0.2 M NaOH was used in the alkali seal. Wild type and vestigial wing discs are inhibited comparably by 10^{-4} M and 10^{-5} M cyanide (Table I). The respiration of wild type whole embryos is inhibited to about the same extent. Sodium azide is also an effective inhibitor of the respiration of wild type and vestigial wing discs. The inhibition varies from about 30 per cent with 0.01 M azide to about 80 per cent with 0.1 M azide (Table I).

2. *Inhibition by Naphthoquinones.*—The inhibitory action of certain 2-hydroxy-3-alkylnaphthoquinones on respiration was observed by Wendel (12) and investigated by Ball, Anfinsen, and Cooper (13) and Anfinsen (14). They found that these naphthoquinones are very potent general respiratory poisons

TABLE I

The Inhibition of Drosophila Respiration by Cyanide and Azide

Solution in diver bulb	Wild type wing disc		Vg wing disc		Wild type embryo	
	Average Q_{O_2}	Inhibition	Average Q_{O_2}	Inhibition	mμl.O₂/ hr./embryo	Inhibition
		per cent		*per cent*		*per cent*
Buffer alone.	20.0	—	9.4	—	46.5	—
10^{-4} M KCN	2.7	86	1.9	79	6.2	87
10^{-5} M KCN	8.0	40	4.1	55	20.7	55
0.1 M azide	4.3	78	1.9	79		
0.05 M azide	5.4	73	2.4	73		
0.013 M azide	9.6	52	5.0	45		
0.01 M azide	13.3	33	6.5	28		

and that they act just below cytochrome c in the chain of respiratory enzymes. Solutions of two of these, SN-5949 (2-hydroxy-3-(2 methyl octyl)-1,4-naphthoquinone) and SN-5090 (2-(3-cyclohexylpropyl)-3-hydroxy-1,4-naphthoquinone), were made up by dissolving a weighed amount in absolute alcohol, evaporating an aliquot of this solution to dryness on a water bath, dissolving the residue in a drop of 0.1 N NaOH, and making up to the proper concentration with phosphate-Locke solution. The concentrations given in Table II are the final concentrations of the naphthoquinones in the phosphate-Locke solution used in the bottom drop. Solutions were made fresh daily from the alcohol solution. The inhibitions found with wild type and vestigial wing discs are given in Table II. These are of the same order of magnitude as those found by Ball, Anfinsen, and Cooper for malaria parasites, liver slices, and yeast suspensions, and by Anfinsen for *Arbacia* eggs.

3. *Stimulation by Ascorbic Acid.*—The results of the cyanide and azide experiments indicated that disc respiration was mediated by an iron or copper

porphyrin system, probably cytochrome-cytochrome oxidase. Since hydro-
quinone, *p*-phenylenediamine, and ascorbic acid will serve as substrates for
cytochrome c, the effects of additions of these substances were investigated.
In all experiments with these easily oxidized substances, solutions were made
up fresh just before use. An extra diver containing buffer and substrate alone
was included with each set of determinations and the autooxidation of the sub-

TABLE II

The Inhibition of Drosophila Respiration by Hydroxynaphthoquinones, Expressed as Per Cent of Normal Respiration

Concentration of inhibitor in solution in diver bulb	SN 5949		SN 5090
	Wild type wing disc	Vestigial wing disc	Wild type wing disc
3.3×10^{-5} M	95	90	87
1.6×10^{-5} M	95	95	82
3.3×10^{-6} M	74	71	51
1.3×10^{-6} M	43	50	29

TABLE III

The Effect of Added Substrates on Drosophila Wing Disc Respiration

Substrate added	Average Q_{O_2} values corrected for autooxidation of substrate		
	Wild type wing disc	Vestigial wing disc	Miniature wing disc
Buffer alone.............................	20.0	9.4	18.3
0.0028 M ascorbic acid...	25.2	33.2	26.4
0.004 M ascorbic acid......	22.7	21.2	19.2
0.002 M *p*-phenylenediamine....	20.2	13.7	
0.0035 M *p*-phenylenediamine........ . . .	19.6	16.7	
0.007 M *p*-phenylenediamine..	19.0	10.6	16.3
0.0035 M hydroquinone....	6.0	11.6	
0.007 M hydroquinone......	0	0	

strate measured in this way was subtracted from the values obtained for the
divers containing buffer, substrate, and disc. It can be seen from Table III
that ascorbic acid causes an increase in the respiration of vestigial wing discs
up to or above the normal value for wild type discs, indicating that the cyto-
chrome c and cytochrome oxidase of vestigial wing discs are normal. There is
also an increase in the respiration of wild type wing discs when ascorbic acid is
added, which indicates that the cytochrome system is not the limiting factor in
the normal respiration of the discs. The respiration of miniature wing discs is
increased over the normal value by the addition of ascorbic acid. The addi-

tion of p-phenylenediamine to the buffer solution causes a lesser increase in the respiration of vestigial wing discs so that the resulting Q_{O_2} lies between the normal value for wild type and vestigial. p-Phenylenediamine and especially hydroquinone seem to have some toxic effect at the concentrations used. The cytochrome c and cytochrome oxidase activities of both vestigial and miniature wing discs are normal and the effects of both the vg and m genes on the respiration of these discs are not produced by altering these enzymes but occur somewhere below cytochrome c in the respiratory chain.

  4. *Inhibition by Iodacetate.*—Iodoacetic acid is an inhibitor of many different enzymes and may decrease the amount of respiration by interfering with the formation of 3-phosphoglyceric acid from triosephosphate or by inhibiting one of the dehydrogenases. Since the action of vg occurs below cytochrome c, the action of iodoacetate on wild type and vestigial wing disc respiration was in-

TABLE IV

The Inhibition of Drosophila Wing Disc Respiration by Iodoacetate

Substance added	Wild type wing disc		Vg wing disc	
	Average Q_{O_2}	Inhibition	Average Q_{O_2}	Inhibition
		per cent		*per cent*
0.001 M iodoacetate.............	4.0	80	2.8	69
0.0001 M iodoacetate.......	6.4	68	7.0	23
0.001 M iodoacetate + 0.02 M malate.......	8.0	60	4.0	56
0.001 M iodoacetate + 0.05 M malate........	16.4	18	8.1	10
0.0001 M iodoacetate + 0.01 M malate...	13.1	34	7.5	17
0.001 M iodoacetate + 0.05 M succinate...	1.8	91	2.4	73

vestigated. Iodoacetic acid was weighed out and added to phosphate-Locke solution and the solution was then neutralized with NaOH, using the glass electrode. Iodoacetate inhibits the respiration of both wild type and vestigial discs to a comparable extent, and this inhibition is partially released by malate but not by succinate (Table IV). These results, although supplying some information about the nature of the respiration in imaginal discs, give no clue as to the point of operation of the vg gene in decreasing respiration.

  5. *Experiments with Dehydrogenases.*—The effect of adding the dehydrogenase substrates, succinate, pyruvate, and lactate was next studied. Pyruvate and lactate were added as the sodium salts, succinate was added as the acid and neutralized with NaOH, using the glass electrode. The pH of all solutions was checked with the glass electrode before they were used. The only substance which increased the Q_{O_2} of the vestigial discs was 0.02 M pyruvate, which gave a slight increase, 33 per cent (Table V). The value of 11.9 for the Q_{O_2} on the addition of pyruvate is the average of six determinations. The difference

between the means, 2.5, has a standard error of 0.70 and thus lies between the 5 per cent and 1 per cent levels of significance.   0.05 M pyruvate is apparently toxic and reduces respiration considerably.   Neither succinate, lactate, nor glucose, when added as a substrate to the buffer in the bottom drop of the diver, increased the respiration of vestigial wing discs.

DISCUSSION

These experiments imply that the biochemical effects of genes may be localized in certain tissues just as the morphological effects are.   Thus the vg and m genes decrease respiration in wing discs but not in leg discs (6), and B and BB genes increase respiration in eye discs but not in wing discs (15).   The four-jointed gene, however, decreases the metabolism of both leg and wing discs, which may be correlated with the fact that although the primary effect of fj

TABLE V

Effects of Added Substrates on Drosophila Wing Disc Respiration

Substrate added	Wild type wing disc Average Q_{O_2}	Vg wing disc Average Q_{O_2}
Control, buffer alone..	20.0	9.4
0.01 M succinate..	14.1	7.3
0.05 M succinate..	15.5	9.5
0.01 M pyruvate.	18.2	10.3
0.02 M pyruvate.	11.5	11.9
0.05 M pyruvate	7.5	4.5
0.02 M lactate	18.3	9.5
0.2 M glucose	16.7	9.1

is on the legs, decreasing the number of tarsal segments from five to four, the wings are also affected and have a shorter, broader shape.

These differences between the respirations of different types are real, due to intrinsic differences in cell metabolism, and not to differences in the types of cells present; e. g., admixtures of various amounts of connective tissue cells. The histological and embryological studies of Chen (2), Robertson (16), Auerbach (3), Steinberg (17), and others show that the same sort of primitive, undifferentiated cells is found in the various discs during the larval period.   Nor are these differences due to the presence in certain discs of deposits of lipid or other metabolically inactive substances; cytologically the cells are similar and free of fat deposits.

The inhibition of the respiration of whole embryos as well as the separate imaginal discs by cyanide and azide indicates that an iron or copper porphyrin system, probably cytochrome-cytochrome oxidase, is present.   With both cyanide and azide the inhibition of respiration is usually not complete, but

approaches 90 or 95 per cent, suggesting that there may be a "cyanide-insensi-tive" fraction of respiration in the discs as there is in many other types of proto-plasm.

The inhibition of disc respiration by the naphthoquinones is further evi-dence that the respiratory pathway in *Drosophila* is similar to that of yeast suspensions, malaria parasites, and liver slices and *via* cytochrome-cytochrome oxidase. Ball, Anfinsen, and Cooper (13) present evidence that these naph-thoquinones inhibit an enzyme lying between cytochrome b and c in the respiratory chain. The order of magnitude of the concentration of naphtho-quinone necessary to produce inhibition of *Drosophila* respiration (0.5 to 1.0 mg./liter) is similar to that found by Ball, Anfinsen, and Cooper for the inhi-bition of respiration in liver slices, yeast suspensions, and malaria parasites, as well as for the inhibition of succinate oxidase *in vitro*, and by Anfinsen (14) for the inhibition of respiration in *Arbacia* eggs. The naphthoquinone SN 5949 is more potent than SN 5090 in inhibiting *Drosophila* disc respiration as it is in inhibiting the respirations investigated by Ball, Anfinsen, and Cooper.

Since ascorbic acid and *p*-phenylenediamine are known to be substrates for cytochrome c, the stimulation of imaginal disc respiration by these substances is additional evidence that cytochrome oxidase and cytochrome c mediate respiration in *Drosophila* discs. The fact that the respiration of vg and m wing discs becomes as great or greater than wild type discs on addition of ascorbic acid shows that the cytochrome c-cytochrome oxidase systems of vg and m are normal and that the effect of the vg and m genes lies somewhere be-low cytochrome c in the respiratory chain. The cytochrome system of wild type discs is normally not used to capacity since the addition of ascorbic acid to the substrate increases the respiration of wild type discs 25 per cent or more. The stimulation of respiration on the addition of ascorbic acid is greatest in the first half hour and falls off gradually after that time. The values for respira-tion given in Table III are averages for respirations over a 1-hour period.

The inhibition of respiration by iodoacetate and its partial release by malate but not by succinate is the same for wild type and vestigial discs. These facts thus do not aid in the identification of the particular enzyme system affected by the vg gene, but do suggest that the four-carbon dicarboxylic acids are in-volved in disc respiration. Malate was found to reverse the iodoacetate inhi-bition of respiration in the oat coleoptile by Commoner and Thimann (18), although in that organism succinate also reversed the iodoacetate inhibition, but to a lesser extent. Malate, succinate, and fumarate are equally effective in reversing the iodoacetate inhibition of respiration in *Neurospora* (19).

Vestigial wing disc respiration is increased slightly, about 33 per cent, by pyruvate but not by succinate, lactate, or glucose. The respiration of wild type discs is not increased by any of the four substrates. The fact that suc-cinate does not increase respiration and does not release iodoacetate inhibition

may mean that these discs do not have succinic dehydrogenase. In contrast, eye disc respiration is increased markedly by the addition of succinate (15), so the failure to metabolize succinate is not a general characteristic of insect tissue. The increase in vestigial respiration on the addition of pyruvate suggests that the pathway of oxidation-reduction reactions in vestigial wing discs is normal, *i.e.* similar to wild type, and that the enzyme affected by the vg gene is somewhere below this. The vg gene may not affect any enzyme in the oxidation-reduction system, but one in a completely different system, such as one involved in protein synthesis, and thereby decrease the utilization of energy and the consumption of oxygen.

When an attempt is made to correlate these physiological and biochemical findings with the results of embryological studies, difficulties arise because the embryologists are not agreed as to the effects of the genes involved. The smaller size of the adult structure in both vestigial wings and Bar eyes has been attributed to a lytic action (20–22); the small size of the vestigial wing is explained by Waddington (23) as due to abnormalities in the folding of the wing bud; and Steinberg (24) states that the small size of the Bar eye is due (*a*) to a smaller amount of material being available for the formation of an eye and (*b*) to variations in the fate of cells of the eye disc which may form either the ommatidia of the eye or head chitin. For both vestigial and Bar there are alternate explanations: the small size is due to the destruction or cytolysis of part of a normal sized embryological rudiment, or the small size is due to the fact that the embryological rudiment itself is smaller than normal. Since the respiration of vg wing discs is lower and of B eye discs higher than the corresponding wild type discs, the same embryological explanation can hardly hold in both cases. Probably the best explanations of the effects of vg and B based on embryological studies are those of Goldschmidt and Steinberg. We could then correlate a decrease in respiration (vg) with cytolysis and an increase in respiration (B) with an organ developing from a smaller rudiment. It may be that the processes leading to the formation of head chitin have a higher oxygen consumption than those leading to the formation of ommatidia. This hypothesis would account for Steinberg's findings (24) that the growth curves of wild type and Bar eye discs (based on measurements of area) are the same during the larval period from 36 hours after hatching until pupation and the results reported previously (15) that the respiration of Bar eye discs is markedly greater than that of wild type eye discs.

SUMMARY

The metabolism of the imaginal discs of wild type, miniature, vestigial, and four-jointed varieties of *Drosophila* was investigated using the Cartesian diver ultramicrorespirometer. Wild type and vestigial wing disc respiration is inhibited by cyanide and azide and thus is mediated by an iron or copper por-

phyrin system, presumable cytochrome-cytochrome oxidase. Respiration is also inhibited by certain hydroxynaphthoquinones, believed to inactivate some enzyme between cytochromes b and c. The respiration of the vestigial and miniature wing discs is increased to normal by the addition of ascorbic acid and to a lesser extent by p-phenylenediamine and hydroquinone, hence the cytochrome oxidase and cytochrome c systems of vestigial and miniature wing discs are normal and the effects of these genes are on enzymes below cytochrome c in the respiratory chain.

The respiratory enzymes of the developing imaginal discs of insects are similar to those of a wide variety of cells from bacteria to mammals. The correlation of these biochemical findings with embryological studies of the discs is discussed.

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STUDIES ON THE INTERMEDIARY CARBOHYDRATE METABOLISM OF AQUATIC ANIMALS

I. The Distribution of Acid-Soluble Phosphorus and Certain Enzymes in Dolphin Tissues*

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The differences and similarities between aquatic and terrestial mammals have been the subjects of several investigations (1–3). Studies of the gross and histologic anatomy of the lungs of several marine mammals have disclosed some structural modifications peculiar to these species (4, 5). The problems of adaptation of physical structure to an aquatic environment have been discussed by Kellogg (6) and the physiology of respiration of diving animals has been reviewed by Irving (7).

The mechanisms for controlling the oxygen supply to the tissues and the ability of many marine mammals to undergo long periods of submergence have been of great interest. Irving, Scholander, and Grinnell (8) studied the respiration of dolphins during rest and diving and found that the resting oxygen consumption was less than that for seals but somewhat greater than for man per unit of weight. They reported that the lactic acid content of the muscle tissue of dolphins did not change significantly during the dive and that blood lactate values were not altered during or after diving, in contrast to the results obtained on rats and ducks (9) and on seals (8) in which lactic acid increased in the muscle during diving and in the blood during recovery.

With some information on the over-all physiology of the dolphin at hand, it was of interest to study the intermediary cellular metabolism, since fundamental differences in the metabolism of aquatic and terrestial mammals must certainly reflect variations in enzymatic processes. Except for the findings of Manery, Welch, and Irving (10) that excised seal skeletal muscle can glycolyze to the same extent as rabbit muscle, data on the concentrations of the intermediates of glycolysis and on the respiratory and glycolytic enzymes in the

tissues of aquatic mammals are lacking.   The present study was therefore carried out to obtain information concerning the glycolysis and respiration of dolphin tissues, since such information might be of value in understanding the energy-yielding reactions of mammals in an aquatic environment.

The present communication gives the results of measurements of the distribution of acid-soluble phosphorus compounds and of some enzymes in several tissues from three adult long-snouted dolphins (*Stenella plagiodon*) and one new-born spotted dolphin of the same species.

<center>EXPERIMENTAL</center>

Collection of Material.—Three adult long-snouted dolphins were harpooned in the coastal waters near Marineland, Florida, and were placed overnight in large tanks at the Marine Studios.[1]   The minor wounds inflicted by harpooning did not impair the normal activity of the animals.   On the following day the animals were anesthetized with pentobarbital sodium (40 mg. per kilo) and the tissues were removed for analyses.   The removal and freezing of all of the tissues from each animal required about 7 minutes.   One new-born animal was obtained at the time of birth and the tissues from this dolphin were also taken for analyses.   The heart of the new-born animal was functioning at the time of birth but respiration had not yet begun.   About 20-gm. samples of liver, kidney, heart, brain, and skeletal muscle (rectus abdominis) were quickly taken by two of us while a third investigator rapidly froze the tissues between two blocks of dry ice.   The samples were then placed in unbreakable cellulose nitrate containers of about 80 cc. capacity, and the vials were covered with rubber caps.   The vials were then placed in wide mouthed thermos jars of pint capacity and the thermos jars were filled with crushed dry ice, stoppered, packed in corrugated cartons containing crushed dry ice, and shipped to the Chicago laboratories by Air Express.   This technique was essentially the same as that devised by Eichelberger, Fetcher, Geiling, and Vos (1) for the collection and shipment of blood and milk from dolphins.   The samples arrived at the laboratories 15 to 18 hours after dissection, remaining frozen throughout transit and until completion of all of the analyses which were begun immediately after arrival of the samples.   After the tissues were prepared for shipment further dissection of the animals was performed to obtain the total weights of the organs of each animal.

Chemical Methods.—The enzyme assays were carried out on homogenates (11) prepared from the frozen tissues.   The homogenization technique for enzyme assays was particularly suitable for the present study since the possibility of using preparations which require intact cells was obviated by cellular damage which necessarily resulted from freezing the tissues.   Furthermore, the use of homogenized tissues allows for an estimation of the maximum activity (12) of the particular enzyme sys-

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[1] The collection of these specimens was made possible through the whole-hearted cooperation of the scientific and technical staffs of the Marine Studios.   The efficient service of the Delta and the Eastern Airlines and the Air Express of the Railway Express Agency made possible the rapid transportation of the frozen tissues from St. Augustine, Florida, to the laboratories in Chicago.

tem to be studied provided the necessary cofactors are added in excess to the system. In the present studies cytochrome oxidase and succinic dehydrogenase were measured by the method of Schneider and Potter (13), malic dehydrogenase by the procedure of Potter (14), and adenosine triphosphatase by the method of DuBois and Potter (15). Measurements of the rate of anaerobic glycolysis and respiration of skeletal muscle, liver, and brain were carried out on tissue homogenates by the procedure of Reiner (16). To test whether the freezing and the time elapsing between the removal of the tissues and the assays influenced the results obtained on dolphin tissues, we carried out control tests with rat tissues which were frozen and stored for a similar length of time and then assayed. Since the results obtained on fresh rat tissues and frozen ones showed no significant differences we believe that the values obtained for the dolphin tissues closely approximate those which would have been obtained if the tissues were assayed immediately after removal from the animals.

The analyses of the acid-soluble phosphorus compounds were carried out according to the scheme outlined by LePage and Umbreit (17), with the exceptions that coenzymes were measured according to Melnick and Field (18) and glycogen was measured on separate samples by the procedure of Good, Kramer, and Somogyi (19).

RESULTS

The Distribution of Cytochrome Oxidase, Succinic Dehydrogenase, and Malic Dehydrogenase in Dolphin Tissues.—Measurements of the cytochrome oxidase, succinic dehydrogenase, and malic dehydrogenase activities of liver, kidney, brain, skeletal muscle, and cardiac muscle of dolphins were carried out to obtain an index of the concentration of some of the respiratory enzymes in dolphin tissues. The results of these assays are shown in Table I. In this and subsequent tables dolphin 1 refers to an adult male, dolphin 2 to a lactating female, dolphin 3 to a young adult male, and dolphin 4 to a new-born dolphin. For comparison of the enzyme activities of tissues from the aquatic mammals employed in these studies with the enzyme activities of tissues from a terrestial mammal, data for adult rats, the only terrestial mammal for which values have been obtained for these enzymes by the same methods, are included in Table I. The values for rat tissues are taken from the data of the investigators who designed the methods used in this study together with results obtained on rat tissues by the same methods in this Laboratory.

The results of these assays demonstrated that all of the dolphin tissues which were examined contained cytochrome oxidase, succinic dehydrogenase, and malic dehydrogenase. As in the case of rat tissues, the greatest cytochrome oxidase activity of dolphin tissues was found in cardiac muscle while the cytochrome oxidase activity of kidney and liver was about one-half as high in dolphin tissues as in rat tissues. Skeletal muscle showed the least cytochrome oxidase activity of the tissues examined and the activity was similar to that of the skeletal muscle of adult rats.

The succinic dehydrogenase activity of dolphin tissues was highest in kidney

and heart muscle and the activity of these two tissues was similar.   Skeletal muscle, brain, and liver exhibited similar succinic dehydrogenase activity, about one-fourth as great as was found in heart and kidney.   The kidney and skeletal muscle of the new-born dolphin (No. 4) had less succinic dehydrogenase activity than the corresponding tissues from the adult animals while heart and liver exhibited activity similar to that of the adult animals.   All of the dolphin

TABLE I

The Cytochrome Oxidase, Succinic Dehydrogenase, and Malic Dehydrogenase Content of Dolphin Tissues

Dolphin No.	Kidney	Brain	Heart	Skeletal muscle	Liver
Cytochrome oxidase (Q_{O_2})					
1	308	186	1330	155	167
2	324	290	1000	190	294
3	302	236	790	174	375
4	181	—	525	57	162
Adult rats..........	695	420	1699	180	479
Succinic dehydrogenase (Q_{O_2})					
1	86.2	25.2	81.4	22.0	21.6
2	70.4	17.3	82.5	18.6	41.1
3	62.5	18.7	64.0	15.0	27.0
4	28.5	—	71.0	6.0	27.0
Adult rats..........	195	48.7	219.0	35.5	87.7
Malic dehydrogenase (Q_{O_2})					
1	39.6	22.4	65.0	13.8	14.0
2	59.2	18.5	53.0	16.2	28.9
3	15.5	13.6	50.0	9.8	25.0
4	18.0	—	44.0	6.0	33.0
Adult rats..........	81.4	27.5	128.0	—	103.5

tissues showed a lower succinic dehydrogenase activity than the corresponding tissues from adult rats.   Malic dehydrogenase activity was greatest in the cardiac muscle of dolphins while kidney, brain, and liver exhibited similar activity about one-half as great as that found in heart muscle.   All of the dolphin tissues contained less malic dehydrogenase than the corresponding adult rat tissues.   While the malic dehydrogenase activity of the tissues from the new-born animal was lower than that of adults the difference was not as great as in the case of cytochrome oxidase or succinic dehydrogenase.

The Adenosine Triphosphatase Activity of Dolphin Tissues.—The adenosine triphosphatase activity of dolphin tissues was measured to obtain an indication of the concentration of a glycolytic enzyme in dolphin tissues. The assays were carried out in the absence of a metallic activator and with calcium at a final concentration of 0.003 M as the activator. It was also of interest to measure the rate of hydrolysis of ATP by homogenates of dolphin tissues using

TABLE II

The Adenosine Triphosphatase Activity of Dolphin Tissues

Activator	Dolphin No.	Heart	Skeletal muscle	Liver	Brain	Kidney
		ATPase units	*ATPase units*	*ATPase units*	*ATPase units*	*ATPase units*
None	1	4.0	7.9	0.9	1.0	1.8
	2	5.0	7.1	1.4	0.9	1.4
	3	3.1	8.0	1.4	1.5	1.8
	4	1.7	1.8	1.8	—	2.2
Calcium	1	5.5	8.4	3.8	1.5	4.9
	2	5.3	7.6	3.0	1.3	2.4
	3	7.4	11.4	6.0	1.5	6.0
	4	5.7	3.8	—	—	—
Magnesium	1	7.5	14.0	8.9	5.6	12.5
	2	8.5	11.1	9.6	4.6	4.9
	3	11.1	18.3	9.2	6.6	8.7
Manganese	1	7.3	11.9	6.5	3.8	6.9
	2	5.3	7.9	4.7	2.5	2.6
	3	10.0	13.9	7.5	5.0	7.2
None	Adult rats	4.8	12.5	3.9	2.4	3.5
Calcium		27.3	23.3	12.9	7.0	20.3

magnesium and manganese in place of calcium. For these tests a final concentration of 0.003 M manganese and magnesium was employed in the adenosine triphosphatase test system (15). The results of these assays are shown in Table II in which all of the values are expressed as micrograms of phosphorus liberated from ATP in 15 minutes by 1 mg. of fresh tissue (adenosine triphosphatase units).

As may be seen from the data in Table II all of the dolphin tissues possessed the ability to catalyze the liberation of inorganic phosphorus from ATP. However, a significant difference was observed between dolphin tissues and rat tissues in the activating effect of calcium. Whereas calcium produces a marked

stimulation of the adenosine triphosphatase activity of rat tissues only a small augmentative effect was noted with calcium in the skeletal muscle, liver, and kidney of dolphins and no significant increase was observed in heart muscle and brain. Of the dolphin tissues skeletal muscle possessed the greatest adenosine triphosphatase activity. However, all of the dolphin tissues exhibited a lower activity of this glycolytic enzyme than the corresponding tissues from adult rats.

Magnesium was a more effective activator than calcium for the hydrolysis of

TABLE III

Anaerobic Glycolysis and Respiration of Dolphin Tissues

Dolphin No.	Tissue	Anaerobic glycolysis $(Q_L^{N_2})$		Oxygen consumption (Q/O_2)	
		Mg	Mn	Mg	Mn
1	Liver	20.2	39.4	15.7	14.1
2		26.0	57.8	10.5	6.2
3		19.2	40.8	5.5	4.0
4		31.8	39.4	4.9	5.3
1	Skeletal muscle	33.6	28.6	5.2	10.3
2		49.0	41.9	5.5	4.3
3		78.4	63.6	3.1	4.2
4		32.1	44.5	0.4	1.9
1	Brain	24.2	31.3	22.6	10.8
2		28.6	27.0	13.4	8.0
3		26.7	26.2	12.1	12.6
Adult rats	Liver	24.0	13.0	12.0	12.0
	Skeletal muscle	103.0	51.0	4.0	7.0
	Brain	66.0		20.0	8.0

ATP by homogenates of dolphin tissues. Manganese acted similarly to magnesium with the activating effect being slightly less than that produced by the same concentration of magnesium.

Respiration and Glycolysis of Dolphin Tissues.—Reiner (16) has recently devised a test system which facilitates the measurement of glycolysis and respiration of tissue homogenates. This method was applied to brain, skeletal muscle, and liver of three adult dolphins using both magnesium and manganese as activators. The results of these measurements are shown in Table III which includes comparable data on tissues from adult rats.

These results indicate that the rate of respiration was lowest in skeletal muscle, although anaerobic glycolysis of skeletal muscle proceeded at a rapid

rate. Manganese and magnesium were of similar effectiveness as activators except for anaerobic glycolysis of liver, where manganese was the more effective; and skeletal muscle, where, except for the new-born dolphin tissues (No. 4), magnesium produced more activation than manganese. The respiratory system with magnesium activation was poorest in the tissues of the new-born dolphin and the immature adult (No. 3) while the glycolytic system was of a similar order of magnitude in the tissues of all four dolphins. There was, in general, a greater disparity between the dolphin and rat values for glycolysis and respiration than for the individual enzymes tested.

Distribution of the Phosphorylated Intermediates of Glycolysis in Dolphin Tissues.—The assays for certain enzymes in dolphin tissues suggested that intermediary carbohydrate metabolism proceeds through similar pathways in aquatic and terrestial mammals. Measurements of the distribution of the phosphorylated intermediates of glycolysis in dolphin tissues were, therefore, of interest to ascertain whether a phosphorylative glycolysis occurred in the tissues of this species and to note the concentrations of the phosphorylated esters in the tissues of this aquatic species. The results of these measurements are given in Table IV in which average values for the compounds in the tissues of the three adult dolphins are given together with the maximum and minimum values for the group.

The data in Table IV on the distribution of acid-soluble phosphorus in dolphin tissues show that all of the tissues contained the phosphorylated intermediates of glycolysis. A comparison of the results obtained here with those obtained by LePage (20) on adult rats shows the similarities and differences between the concentrations of phosphorylated compounds in the tissues of the two species.

The skeletal muscle of dolphins and rats contains similar quantities of inorganic phosphorus, total phosphorus, nucleotides, and phosphoglyceric acid. The fructose phosphate esters were much higher in quantity, and the quantity of phosphocreatine about one-half as great in dolphin skeletal muscle as in rat muscle. Cardiac muscle also contained similar quantities of most of the compounds as compared with rat heart muscle. However, the fructose phosphate esters were again higher in amount than in the cardiac muscle of rats. Brain contained less inorganic phosphorus, total phosphorus, phosphocreatine, phosphoglyceric acid, and pyridine dinucleotides than did rat brain. The concentration of phosphorylated compounds in the liver of dolphins was somewhat lower but within the same general range as in rat liver. The kidney tissues of dolphins, however, presented a somewhat different picture as compared with rats since all of the compounds were present in much lower quantities than in rat kidneys.

A fractionation of the acid-soluble phosphorus of the tissues from the new-born dolphin was also carried out. Skeletal muscle contained about one-half the quantity of inorganic phosphorus, total phosphorus, ATP, and phospho-

creatine while the other constituents were present in about the same quantity as in adult dolphins. The values for the phosphorylated intermediates in the other tissues of the new-born animal were of the same order of magnitude as

TABLE IV

The Distribution of Acid-Soluble Phosphorus in Dolphin Tissues

Tissue	Inorganic phosphorus	Organic phosphorus	ATP	ADP	Adenylic acid	Phospho-creatine	Phospho-glyceric acid	Hexosedi-phosphate
	μM/100 gm.	*μM/100 gm.*	*μM/100 gm.*	*μM/100 gm.*	*μM/100 gm.*	*μM/100 gm.*	*μM/100 gm.*	*μM/100 gm.*
Skeletal muscle	941 (734–1260)	4230 (3940–4400)	437 (346–595)	38 (10–55)	120 (90–142)	600 (570–640)	216 (190–250)	221 (169–265)
Brain	729 (660–820)	890 (675–940)	113 (95–128)	13 (10–16)	27 (20–35)	44 (20–90)	48 (45–50)	38 (34–93)
Liver	1162 (1100–1236)	1454 (1340–1570)	50 (40–62)	115 (100–132)	68 (46–82)	73 (58–90)	65 (50–79)	51 (41–58)
Kidney	749 (669–780)	504 (455–590)	7 (4–11)	2 (0–6)	11 (7–15)	53 (20–91)	18 (16–20)	48 (31–63)
Heart	1280 (1046–1520)	2175 (1925–2325)	223 (190–290)	28 (22–32)	82 (68–90)	168 (125–220)	114 (96–142)	90 (82–100)

	Glucose-1-PO₄	Glucose-6-PO₄	Fructose-6-PO₄	Triose-PO₄	Coenzymes	Pentose-PO₄	Phosphopy-ruvic acid
	μM/100 gm.	*μM/100 gm.*	*μM/100 gm.*	*μM/100 gm.*	*μM/100 gm.*	*μM/100 gm.*	*μM/100 gm.*
Skeletal muscle	283 (212–385)	530 (449–631)	233 (187–286)	9 (6–12)	96 (83–107)	116 (101–146)	67 (40–90)
Brain	45 (29–71)	76 (60–95)	51 (33–75)	0	19 (15–24)	56 (44–86)	0
Liver	143 (81–230)	246 (158–300)	69 (59–87)	0	60 (58–62)	113 (85–130)	0
Kidney	101 (71–141)	58 (50–71)	54 (37–74)	0	14 (12–16)	12 (9–15)	0
Heart	53 (42–63)	336 (227–400)	134 (125–216)	9 (6–12)	65 (57–75)	157 (105–198)	9 (2–25)

for the adult animals, closely approaching the minimum values obtained on the adult animals.

Glycogen Content of Dolphin Tissues.—Glycogen values were obtained for all of the tissues from the adult animals and from three of the tissues from the new-born dolphin. The results of these measurements are shown in Table V.

Of particular interest in these measurements was the high glycogen content of skeletal muscle. As shown in Table V the glycogen of skeletal muscle of the dolphins was above 0.6 per cent in every case with the average for the four ani-

mals being 0.98 per cent whereas the average value for adult rats was 0.16 to 0.20 per cent under comparable conditions of fasting for 24 hours. The low level of glycogen in the dolphin livers was probably due to fasting from the time of capture until death (24 hours).

TABLE V

The Glycogen Content of Dolphin Tissues

Dolphin No.	Glycogen				
	Skeletal muscle	Heart	Liver	Brain	Kidney
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
1	0.895	0.12	0.08	0.05	0.10
2	0.630	0.05	0.07	0.017	0.05
3	0.809	0.24	0.88	0.012	0.08
4	1.570	0.01	0.16	—	—

TABLE VI

Organ Weights of Dolphins

Organ	Dolphin 1 (adult male)		Dolphin 2 (adult female)		Dolphin 3 (young male)		Average per cent of body weight	
	Weight	Per cent of body weight	Weight	Per cent of body weight	Weight	Per cent of body weight	Dolphin	Rat
	kg.		*kg.*		*kg.*			
Total body weight.. ...	139		110		90			
Blubber..............	22.7	16.3	17.4	15.8	18.1	20.1	17.4	—
Gastrointestinal tract and contents........	7.0	5.04	7.73	7.03	5.0	5.55	5.87	—
Lungs...	—	—	2.11	1.92	2.27	2.52	2.22	0.70
Liver....	2.63	1.89	—	—	1.75	1.94	1.92	3.90
Kidneys	0.955	0.69	0.826	0.75	0.600	0.67	0.70	0.80
Brain...............	0.900	0.65	0.866	0.79	1.06	1.18	0.87	0.85
Thyroid..............	0.023	0.016	—	—	0.013	0.014	0.015	0.005
Testes...............	6.300	4.54	—	—	0.113	0.13	—	—
Heart................	0.682	0.49	0.570	0.52	0.48	0.54	0.52	0.34
Skeleton.............	5.11	3.67	3.86	3.51	3.30	3.66	3.61	—

Organ Weights of Dolphins.—Since data on the weights of individual organs of the dolphin in relation to the total body weight are lacking, we recorded the weights of some of the organs from the three adult dolphins. Such data may be of value in further studies on the metabolism of this species. The results of these measurements are shown in Table VI. For comparison, values for the weights of certain rat organs, as per cent of total body weight, are included.

The most striking differences between the two species are the relative sizes

of the lungs and the liver.   The dolphin lungs occupy over three times as much of the total body weight as do rat lungs, while the rat liver is, in proportion, twice as large as the dolphin liver.   The kidneys and brains of the two species are similar with respect to their weights as compared to the total weight of the animals, while the dolphin heart represents a greater percentage of the body weight than does the rat heart.   The thyroid of the dolphin is also relatively larger than that of the rat; this difference may be largely attributable to the disparity in the temperature of the environment of dolphins and rats.

DISCUSSION

These studies on the intermediary carbohydrate metabolism of dolphin tissues indicate that the pathways for the dissimilation of carbohydrate and the energy-yielding reactions in this aquatic mammal are similar in many respects to those of terrestial mammals.

The assays for cytochrome oxidase, succinic dehydrogenase, and malic dehydrogenase demonstrated the presence of all of these respiratory enzymes in dolphin tissues.   A considerable difference was noted in the enzyme activities of the individual dolphin tissues, with heart muscle showing the greatest concentration of all of these enzymes as is the case with rat tissues.   The activity of these respiratory enzymes in dolphin tissues was lower than that of the corresponding rat tissues.   Since the oxygen consumption of dolphins is somewhat higher than that for man per unit weight (8), it is unlikely that the lower respiratory enzyme activity in dolphin tissues as compared with rats is due to adaptation for possible diminished oxygen supply, but it is probably rather a reflection of a slower rate of metabolism in dolphins as compared with rats. A comparison of the enzyme activities obtained in dolphin tissues with those of the tissues of large terrestial mammals would be of interest when data on the latter animals obtained by the same methods become available.

The enzyme activity measurements on the new-born animal are the first experiments to be carried out on dolphins obtained at the time of birth.   They demonstrated that the tissues contain much higher enzyme concentrations at the time of birth as compared with adult animals of the same species than do new-born rats (21).   This finding is consistent with the ability of young dolphins to perform all the movements of the adult animals immediately after birth.

The lower respiratory enzyme activity of dolphin brain tissue as compared with rat brain might be responsible for the greater sensitivity of dolphins to barbiturates (1), if barbiturates produce their narcotic action through inhibition of cellular respiration as considerable evidence obtained by Quastel and Wheatley (22) has suggested.

Measurements of the rate of hydrolysis of ATP by the adenosine triphosphatase of dolphin tissues showed, in contrast to results obtained on rat tissues,

that calcium provides very little activation of the adenosine triphosphatase of tissues from this species while magnesium and manganese had a definite stimulatory effect on the hydrolysis of ATP by homogenates of dolphin tissues. The lack of an activating effect by calcium might suggest either that this ion is not the activator for the adenosine triphosphatase of dolphin tissues or that the tissues contained a sufficient quantity of calcium to provide maximum activation of the enzyme. The latter possibility seems unlikely, however, in view of the similarity in the concentration of electrolytes in the skeletal muscle of dolphins and terrestial mammals which was noted by Eichelberger, Geiling, and Vos (3). To our knowledge the activation of adenosine triphosphatase by magnesium rather than by calcium has been reported (23) in only one other tissue, the electric organ of the electric fish (*Torpedo*).

Glycolysis was shown to take place in dolphin tissues, by manometric measurements of acid formation using glucose as the substrate. This finding is in agreement with the demonstration by Irving, Scholander, and Grinnell (8) of lactic acid formation *in vitro* by dolphin skeletal muscle. The presence of the phosphorylated intermediates of the Emden-Meyerhof glycolytic scheme in all of the dolphin tissues examined indicates that the energy-yielding reactions in this aquatic species are qualitatively similar to those in the tissues of terrestial mammals. The high concentration of glycogen in the skeletal muscle of the dolphin indicates the ready availability of the substrate for glycolysis during muscular activity, which is needed for the energy-yielding reactions enabling this species to swim at speeds around 25 knots.

The finding that the enzymatic reactions examined in this study are qualitatively similar in dolphins and in terrestial mammals supports the idea (7) that adaptation in aquatic mammals to prolonged submergence is due to a more highly developed mechanism for differential shunting of oxygen rather than to a shift in intermediary metabolism.

The present studies have demonstrated that it is possible to obtain tissues at localities far removed from the laboratories, and by freezing them immediately in dry ice the enzyme activities can be preserved while the tissues are sent to the laboratories. Such a technique might prove useful in future studies on the intermediary metabolism of animals captured at sites far removed from the equipment necessary for metabolic studies.

SUMMARY

1. Liver, kidney, brain, skeletal muscle, and cardiac muscle from one newborn and three adult long-snouted dolphins (*Stenella plagiodon*) were obtained for enzyme studies.

2. All of the dolphin tissues exhibited cytochrome oxidase, succinic dehydrogenase, and malic dehydrogenase activity. Considerable differences in the enzyme activities of the various tissues were noted, with cardiac muscle exhibit-

ing the highest respiratory enzyme activity.   The enzyme activities of dolphin tissues were lower than those of the corresponding rat tissues.

3. All of the dolphin tissues exhibited adenosine triphosphatase activity which was accelerated by magnesium and manganese but, in contrast to rat tissues, was only slightly activated by calcium.

4. Measurements of the distribution of acid-soluble phosphorus in dolphin tissues indicated that glycolysis in all of the tissues examined proceeded through the Emden-Meyerhof phosphorylation scheme.

5. The average glycogen content of dolphin skeletal muscle was 0.98 per cent as compared with 0.16 to 0.20 per cent for rat skeletal muscle.   The high glycogen content of dolphin skeletal muscle indicates a ready source of substrate for glycolysis even during submergence when the blood supply may be differentially shunted to other organs.

6. Measurements of the organ weights of dolphins showed that the lungs occupy over three times and the liver one-half as much of the total body weight as do these organs in the rat.   The heart and the thyroid gland of the dolphin are also larger in proportion to the total body weight than in the rat while the relative weights of the other tissues in the two species are about the same.

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THE REACTION BETWEEN ACTOMYOSIN AND ADENOSINE TRIPHOSPHATE*

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I

INTRODUCTION

The modern tendency in the biochemistry of muscle is to consider the enzymatic dephosphorylation of adenosine triphosphate (ATP) as the energy-yielding reaction of muscular activity. In a preceding paper by Mommaerts and Seraidarian (16) it was demonstrated that there are serious objections to this viewpoint, which currently seems to be untenable. The main experimental basis for this conclusion was the demonstration that the hydrolysis of ATP by myosin-ATPase can account at most for only a small percentage of the speed of breakdown of ATP in contracting muscle as actually observed.

Even before this development, the criticism could have been made that the proponents of what may be called the myosin-enzyme theory failed to make the mechanism of the energy transfer clear. It is stated repeatedly (e.g. Dainty et al., 9) that the reaction energy of the hydrolysis of ATP, supposedly of the order of magnitude of 10,000 calories per mol phosphate, is somehow given over to the myosin, but no explanation of the mechanism of this transfer has been attempted.

Extremely valuable contributions have been made by A. Szent-Györgyi (23), independently of any theory. He showed that myosin reacts with ATP, and that various physical changes can be evoked in actomyosin as a result of this reaction. In an earlier publication (12) the author showed that this reaction is actually a combination between ATP and myosin, but the methods were imperfect and the mechanism was not worked out in detail.

It is the purpose of this paper to present a preliminary analysis of the combination between ATP and myosin, and of some of the physical phenomena resulting from it. The most striking of the effects discovered by Szent-Györgyi is certainly the contraction of actomyosin threads under influence of ATP (22, 23). For several reasons, however, this phenomenon is not well suited to

quantitative analysis. More promising seems the effect of ATP upon the viscosity of actomyosin solutions (Banga and Szent-Györgyi, 3), a phenomenon studied by the author in previous approaches (12–14). This problem is apparently linked with that of the form and dimensions of the myosin molecules (13, 15) and with that of the forces causing their aggregation (14). These aspects will be reconsidered in the future. The present paper is devoted to a more formal study of the reaction between ATP and myosin, without any attempt to consider in detail the molecular mechanism and its implications.

As discovered by Banga and Szent-Györgyi (3), a solution of actomyosin with a sufficient actin content has a considerably higher viscosity than a solution of pure myosin, or than a solution of actomyosin with less actin. Upon the addition of ATP the viscosity of every actomyosin solution drops approximately to that of pure myosin. The difference in viscosity between actomyosin and myosin has been ascribed by the author (13) to the fact that the myosin molecules in actomyosin are aggregated in an end-to-end arrangement. The effect of ATP on the viscosity of actomyosin is then due to a dissociation of this complex protein (14). Somewhat similar effects have been observed by Dainty *et al.* (9), and were interpreted by them as a contraction of the dissolved myosin molecules. This interpretation was disproved in an earlier paper (14).

Although at present it is difficult to relate this viscosity effect directly to the problems of contraction, the effect seems to offer an advantageous approach to the study of the reaction between ATP and myosin. The main advantage is that the phenomenon can be studied in solution, and that it can be measured quantitatively.

II

General Description of the Observed Effects

In this study the viscosity was measured in viscosimeters of the Ostwald type. Measurements were done at 0°, or at temperatures between 16° and 24°. In most cases the actomyosin used was actomyosin B, prepared as described in an earlier paper (16). The viscosimeter usually contained 5 cc. of a solution of actomyosin in 0.5 M KCl, each cubic centimeter of the solution averaging 2 to 3 mg. protein per cc. ATP and other reagents were added through a capillary pipet, with complete mixing. They were dissolved in small enough amounts of fluid, 0.05 cc., that the dilution due to this addition did not in itself decrease the viscosity to a measurable degree.

The results of a series of experiments done with different amounts of ATP are given in Fig. 1. It is seen that the viscosity drops immediately after addition of the ATP, as far as can be judged by these experiments (no rapid technique has been devised yet, but the reaction certainly needs no more than a few seconds, probably much less). After some time, varying directly with the amount of ATP added, the effect of the reaction diminishes gradually, ap-

parently through hydrolysis of the ATP. With certain precautions it is pos-
sible to do the experiments in such a way that the original viscosity returns
quantitatively. In experiments carried out with the routine technique, how-
ever, there usually was a residual effect: the viscosity did not return entirely
to the original value. The magnitude of this difference is variable. The
experiments of Figs. 1 and 2, done in the summer, constitute extreme examples.
This effect is understandable when one realizes that after the splitting of ATP
the actomyosin aggregates again, although under very different circumstances
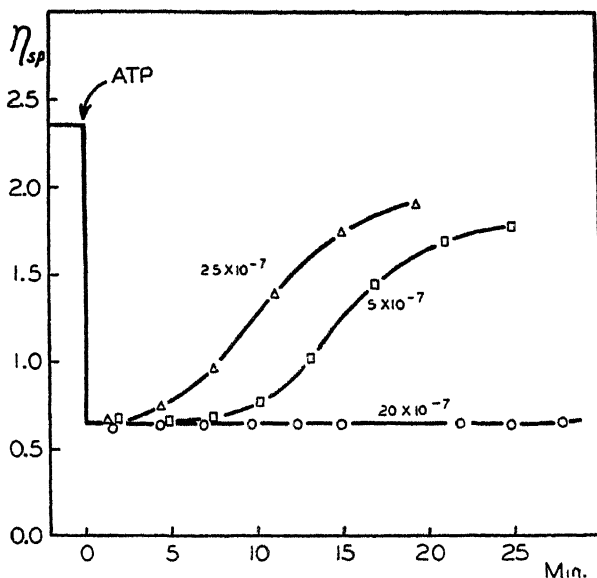
FIG. 1. Effect of ATP upon the viscosity of actomyosin solutions, containing 12.5
mg. protein in 5 cc. Temperature 0°C. ·

than when it was formed during the extraction of muscle. Actomyosin is
certainly polydispersed, with respect to its degree of aggregation. The variation
among the different individual aggregates may depend on the way in which they
were formed. After the addition and removal of the ATP, the micellar struc-
ture of the actomyosin, and thereby the viscosity of its solution, have changed.
A more complete analysis of this problem, in terms of skew or polymodal poly-
disperity, may be attempted at another time. In anticipation it may be said
that the phenomenon indicates that the aggregates in actomyosin have a per-
manent character. This would mean that the high viscosity and other proper-
ties of actomyosin are not due to long range intermolecular forces, as assumed
by Bernal and Fankuchen (4) for tobacco mosaic virus.

Returning to Fig. 1, we can describe the composite behavior shown in the

curves in terms of two separate reactions: the initial "viscosity response" and the subsequent "recovery effect." This recovery effect must be explained by the enzymatic hydrolysis of the ATP by the myosin, which has enzymatic activity in 0.5 M KCl solution (23, 16). The speed of this effect is diminished considerably at low temperatures.
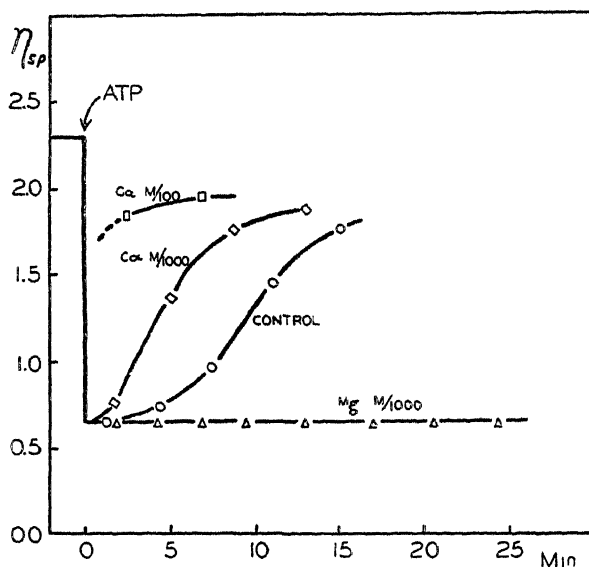
FIG. 2. Effect of ATP upon the viscosity of actomyosin solutions, as affected by Ca and Mg. Experimental data as in Fig. 1; 2.5 × 10^{-7} mol ATP added.

III

The Effect of Calcium and Magnesium

The study of these ions is indicated because of their profound influence upon the enzymatic activity of myosin-ATPase, and still more because of their effects upon contractility of muscle and of actomyosin threads. It may be remembered that enzymatic activity is promoted by Ca and inhibited by Mg (see reference 16) whereas contraction of actomyosin threads is inhibited by Ca and enhanced by Mg (see Szent-Györgyi, 22, 23).

The influence of Ca and Mg ions upon the viscosity effects is shown in Fig. 2 which refers to experiments done at 0°. It is found that, provided sufficient ATP is added, the magnitude of the initial viscosity response is entirely unaffected by the presence of these ions, but that the recovery effect is strongly accelerated by Ca and inhibited by Mg.

This could be taken as an indication that the combination between myosin and ATP which leads to the viscosity response, is not affected by these ions.

However, such a conclusion is not valid, since ATP was in excess in these experiments. A possible promotion by Mg and a possible inhibition by Ca, should reveal themselves only if the available quantity of ATP is not large enough to overcompensate inhibition. Such experiments could not be done with the technique employed in this study, due to the difficulties mentioned in section VI. However, there is already evidence that the primary reaction between ATP and myosin is indeed activated by Mg and inhibited by Ca. In fact, the position of the curve referring to a $CaCl_2$ concentration of $M/100$ in Fig. 2 has to be explained in this way. These problems will be dealt with in a special publication.

The influence of Ca and Mg upon the recovery effect is at least partly due to the fact that the enzymatic hydrolysis of the ATP by myosin-ATPase is inhibited by Mg and increased by Ca. Thus, in the presence of Ca, the quantity of ATP in the system will be more quickly reduced below an effective minimum needed for a full effect, whereas in the presence of Mg the opposite will occur. This is however, not the only reason. At the same subminimal ATP concentration the effect will be further decreased due to the presence of the inhibiting Ca, whereas in the presence of the promoting Mg the decrease will be entirely or partly compensated. The situation in the case of Mg is further complicated by the presence of myokinase or ADP-isomerase, which reconverts ADP into active compounds (see section IV). In this way the viscosity effect is further protracted.

IV

The Specificity of the ATP Effect

It seems reasonable to suppose that ATP influences myosin through interaction of some part of its molecule with some group of the myosin. The question arises then whether substances similar or related to ATP affect myosin in the same way. This leads to the study of inosinetriphosphate (ITP) in which the purine nucleus is changed; adenosine diphosphate (ADP) and monophosphate (AMP) which contain less phosphorus; and inorganic substances with the pyrophosphate configuration.

ADP was prepared from ATP through enzymatic dephosphorylation with crystallized myosin and purification as Ba salt. ITP was prepared from ATP through deamination with nitrous acid. Inorganic Na triphosphate was put at my disposal through the kindness of Dr. A. Deutsch, Kemiska Institutionen, Lund, Sweden. Pyrophosphate was a commercial preparation.

Adenosine diphosphate (see Fig. 3) has only a very slight influence which might be ascribed to small amounts of admixed ATP. If Mg is added, a strong viscosity response is the result. This differs, however, from the ATP effect in that it requires time to develop fully. The same result can be obtained in
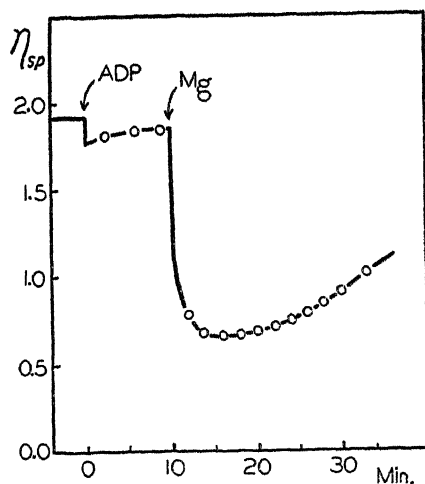
FIG. 3. Effect of ADP (approximately 10^{-6} mol) and Mg ions upon the viscosity of actomyosin solutions (12 mg. protein in 5 cc; temperature 16°).
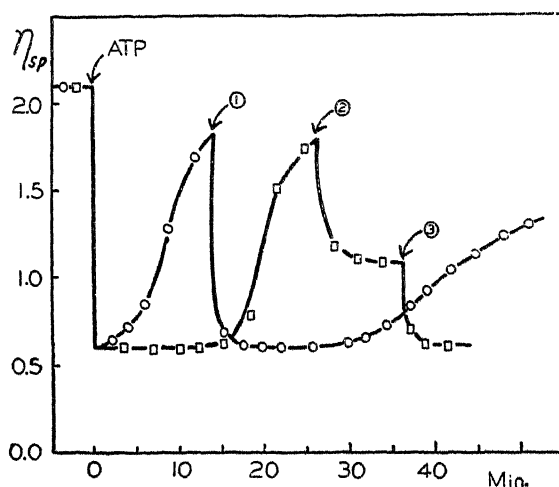
FIG. 4. Effect of addition of Mg after completion of the recovery effect, due to regeneration of ATP from ADP by myokinase. Two experiments, with addition of 5×10^{-7} mol ATP (circles) and 15×10^{-7} mol ATP (squares) (temperature 16°). At 1 and 3, addition of Mg to a concentration of 0.01 M, at 2 to a concentration of 0.001 M (12 mg. protein in 5 cc.).

a somewhat different way by first adding ATP in the absence of Mg (see Fig. 4). After the recovery effect is complete or in other words after the ATP is decomposed to ADP, Mg is added; this causes a second drop in the viscosity, but again this effect is not immediate.

The explanation of these phenomena is that actomyosin, if prepared directly from muscle, is not entirely pure but contains small amounts of an enzyme which in the presence of Mg ions causes a change in the ADP. Whether this must be interpreted as a conversion of 2 ADP into 1 ATP and 1 AMP under influence of myokinase (Kalckar, 10), or as an isomerization of ADP into a form which affects myosin as does ATP (Banga's "ADP-isomerase", 2) can be left undecided. In any case, the phenomenon is restricted to actomyosin B prepared directly from muscle. If myosin is crystallized as such, and combined with actin prepared according to Straub (19, 20), the effect does not occur (compare Szent-Györgyi, 23).
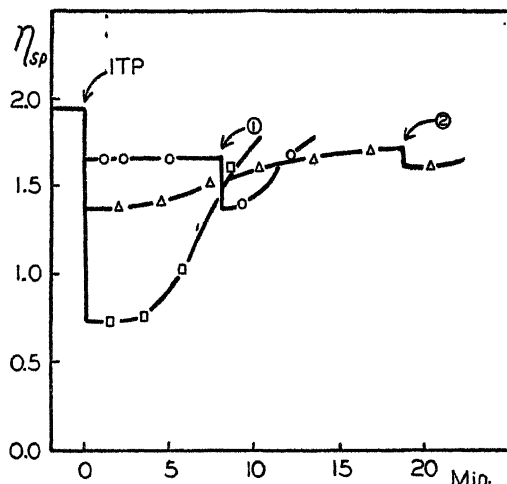
FIG. 5. Effect of ITP on the viscosity of actomyosin (14 mg. in 5 cc.). Lowest curve (squares) in the presence of 0.01 M $MgCl_2$. The other experiments first without Mg; addition of Mg to a concentration of 0.01 M at 1 and 2. Temperature 20°C.

Adenosine monophosphate (Na or K salt of muscle adenylic acid) had no effect under the conditions studied.

Inosinetriphosphate, which is hydrolyzed by myosin-ATPase (Kleinzeller, 11; Mommaerts and Seraidarian, 16) is not able to induce the viscosity response at room temperature, unless Mg is present. The enzymatic splitting of ITP does not require Mg ions. Consequently the magnitude of the effect depends on the time of addition of the Mg (Fig. 5). If added with the ITP, the effect is shown in its maximal degree. If the Mg is added after the ITP, the effect is less, due to the splitting of the ITP which has taken place in the meantime. At low temperature, however, ITP shows activity without Mg ions (Fig. 6), but in this case the reaction is sluggish. As long as the maximal effect has not been reached, it may be enhanced to its final value by Mg. ATP causes no further increase.
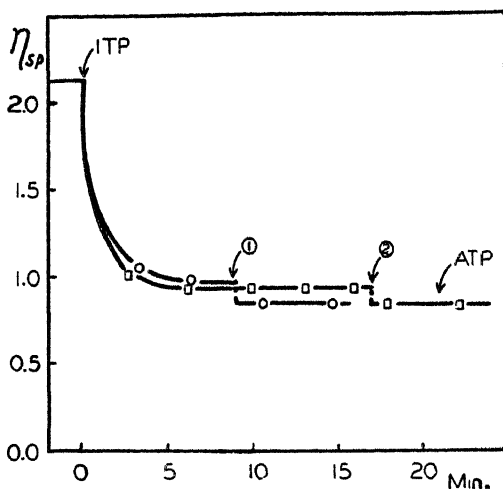
FIG. 6. Effect of ITP at low temperature (0°C.). In the beginning no Mg; addition of MgCl₂ to a concentration of 0.01 M at 1 and 2. Actomyosin 15 mg. in 5 cc.
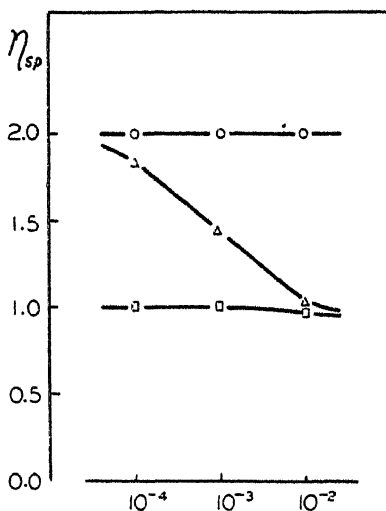
FIG. 7. Effect of inorganic pyrophosphate and Mg on the viscosity of actomyosin (30 mg. in 10 cc.). In all samples, 0.0005 M Na pyrophosphate. Upper curve (circles): before addition of Mg. Middle curve (triangles): after addition of Mg, to the final concentrations indicated on the abscissa. Temperature 17°. Lower curve (squares): the same, after cooling to 0°C.

The behavior of inorganic pyrophosphate is still more complicated. At room temperature, it may induce the viscosity response in the presence of Mg, but unless very high concentrations of Mg are present, the effect remains in-

complete. It takes time to develop fully. At low temperature, 0°C., pyrophosphate reacts more easily. The reactions are almost instantaneous, and the full effect is reached even at low concentrations of Mg. Fig. 7 summarizes the results of an experimental series, in which the viscosities of actomyosin solutions containing 0.0005 M sodium pyrophosphate were measured 30 minutes after the addition of the Mg salt. Then the solutions were cooled and the viscosities were measured again without further waiting. If the measurements at room temperature are made in less than 30 minutes, the differences between these and the results at 0° are still more pronounced. Straub (21)
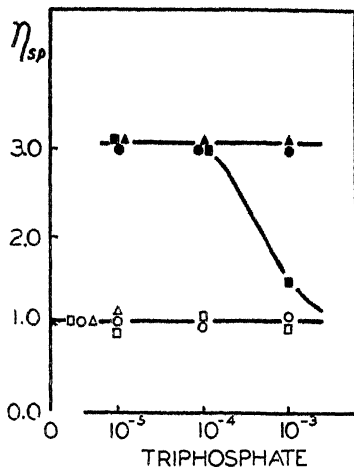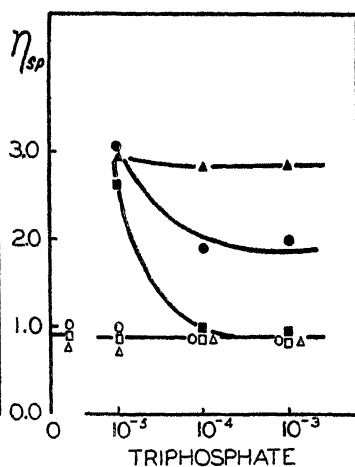
FIG. 8 a   FIG. 8 b

FIG. 8, a and b. Effect of inorganic triphosphate on the viscosity of actomyosin (15 mg. in 10 cc.). Fig. 8 a at 18°, Fig. 8 b at 0°C. Solid symbols: without addition of ATP. Open symbols: after adding ATP in addition to the other substances present. Squares: in presence of $MgCl_2$, 0.01 M; triangles: with $CaCl_2$, 0.01 M; circles: without Mg or Ca.

found that pyrophosphate has an effect at low, but not at high temperatures. If his system contained some Mg, his observations fit well with the above analysis.

Since inorganic pyrophosphate is not decomposed by myosin-ATPase, the viscosity drop is not followed by any recovery effect.

The behavior of inorganic triphosphate, $Na_5P_3O_{10}$, resembles that of pyrophosphate. At room temperature (Fig. 8) the triphosphate has an effect at rather high concentration, if Mg is present. At low temperatures the effects in the presence of Mg are stronger. Sometimes an effect was found at 0° without addition of Mg, but these results were not regularly reproducible. Such reactions seemed to have complicated time relations. In all experi-

ments with Na triphosphate, long reaction times were allowed before the measurements were taken.   The time course of the development of the effects with triphosphate has not yet been investigated.

<div align="center">V</div>

Enzymatically Inactive Actomyosin

With regard to the questions raised in section II concerning the explanations of the nature of the ATP effect it would be of interest to know whether the effect is also possible under conditions which exclude the enzymatic activity of the myosin.   The experiments in the presence of Mg are not sufficient for this purpose.   An attempt was therefore made to prepare actomyosin which would be devoid of any enzymatic activity.

From the literature one would get the impression that this is an easy matter. It is reported that enzymatic activity of myosin preparations can be abolished, by precipitation at or below pH 6 (17) without denaturing the myosin, and also by oxidation with H_2O_2 (24).   Notwithstanding repeated efforts this was never confirmed.   Actomyosin kept its enzymatic activity even on repeated precipitation at pH 5.2, and was not inactivated by H_2O_2 in moderate quantities.

The reason for this difference is probably the total absence of heavy metals in the present experiments.   If common distilled water is used for the preparative work, myosin will gradually accumulate Cu.   The actual concentration of this ion in common distilled water may be very low, but if myosin is repeatedly precipitated with large amounts of water, Cu will accumulate.   Indeed, Bailey (1) could demonstrate the presence of this metal in ashed myosin. As explained in the previous publication (16), heavy metal impurities were carefully excluded during the preparation by the use of water redistilled from an all glass apparatus.

It turned out that it was possible to inactivate myosin-ATPase by the manipulations mentioned above, if traces of Cu sulfate were added.   This procedure was not without difficulties since excessive amounts of the metal caused denaturation.   The inactivation by hydrogen peroxide was studied in some detail.   It was reproducible if H_2O_2, Cu salt, and ATP were present together.   After purification however, the inactivated actomyosin behaved as an actomyosin of diminished actin content.   Apparently part of the actin was destroyed.   If the protein was incubated with Cu salt and peroxide without ATP, the inactivation of the enzyme was very incomplete.   In some experiments an attempt was made to inhibit the ATPase with p-chloromercuribenzoate (kindly made available to me by Dr. Leslie Hellerman of Johns Hopkins University).   Here, very unexpected complications were met with, to which a special investigation will be devoted.

Finally, experiments were done to study the effect of ATP added directly in

the viscosimeter to actomyosin in the presence of Cu and H_2O_2. In one experiment at low temperature, this procedure gave clear cut results. The viscosity response was normal but the recovery effect was entirely absent. Nevertheless, this experiment was still not satisfactory for several reasons. The problem will be followed up again in the near future.

VI

The ATP Dissociation Curve of Myosin

In an earlier study (12) the stoichiometric proportions in which ATP and actomyosin react were investigated. This was done by measuring quantitatively the viscosity-lowering effect of subminimal doses of ATP. These measurements met with great experimental difficulties, since at such low ATP concentrations the recovery effect sets in immediately, and the initial response is much diminished before the first measurement is done. The problem was tentatively solved by extrapolating the viscosity-time curves towards zero time, making certain rather arbitrary assumptions concerning the form of these curves. From such studies it was concluded that myosin reacts with ATP in units with a weight of 10^5 relative to the hydrogen atom, and that ATP and myosin form a sparingly dissociated compound. Similar results were obtained by Straub (18) with actomyosin A under somewhat different conditions.

That the extrapolation procedure was inaccurate was fully realized in 1942, and this became even more obvious during the present study. An attempt was made to improve the experiments by the construction of special viscosimeters with reservoirs of only about 1 cc. and correspondingly short outflow times. However, no real improvement was arrived at in this way.

With the same method, an attempt was made to obtain an ATP-myosin dissociation curve in the presence of 0.02 M $MgCl_2$. Under these circumstances the recovery effect is retarded considerably, and the method becomes much more reliable, although certainly not yet satisfactory. The results of one experimental series are represented in Fig. 9 in the form of a dissociation curve. The abscissa gives the total concentration of ATP in the solution, the ordinate gives the magnitude of the initial viscosity response, expressed as per cent of the maximal effect; it represents at the same time the percentage of the myosin units present as ATP complex. The curve, which is a distinct improvement over the results obtained in 1942, confirms that ATP and myosin form a sparingly dissociated compound. However, the molecular weight of the unit of the molecule which combines with ATP is decidedly more than 100,000. From analysis of the results obtained a unit weight of about 360,000 was computed.

Since in the presence of Mg the recovery effect is so strongly delayed, these

newer results are certainly more reliable than the experiments in the absence of Mg. It should be realized however, that with Mg the situation is not the same as in the absence of this ion.

Further studies with more rapid techniques will be devoted to the analysis of these questions. A quantitative discussion of the results will also be postponed.
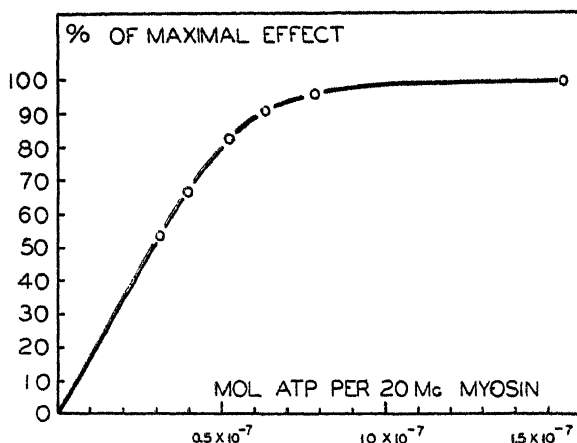
FIG. 9. Actomyosin-ATP dissociation curve in the presence of 0.02 M MgCl₂. Explanation in the text.

VII

DISCUSSION OF THE RESULTS

The main phenomenon studied in this investigation was the decrease of the viscosity which actomyosin solutions undergo after addition of ATP. As described above, these effects have a typical time course, in which one has to distinguish the immediate drop in viscosity, here called viscosity response, and its subsequent reversal here called recovery effect. The latter effect was ascribed to continuously proceeding enzymatic decomposition of the ATP.

It should be said that two fundamentally different explanations of these processes seem possible. On the one hand, it may be supposed that the viscosity response is due to a combination between myosin and ATP, and that the recovery effect is due to a disappearance of the ATP through the action of ATPase. But it is also conceivable that the viscosity response is caused by a continuous transfer of reaction energy from the splitting of ATP towards the myosin. The recovery effect means then that, as the ATP concentration drops, gradually less and less myosin molecules are involved in the reaction. For the first type of explanation it is immaterial whether myosin itself is the ATP-hydrolyzing enzyme or not. The second explanation is intelligible only

if myosin and ATPase are identical. This second interpretation is not in disagreement with the type of ATP-myosin dissociation curve discussed in section VI, since this curve can very well be looked upon as an enzyme-substrate saturation curve.

The current ideas concerning the rôle of ATP and myosin-ATPase, although not developed in detail, belong to the second group of interpretations. They all assume that the enzymatic hydrolysis of ATP by myosin is the cause of muscular activity, and that in this process the free energy liberated in the dephosphorylation of ATP is transferred to the enzymatically active contractile structure. On the other hand, this type of explanation is in disagreement with the results of the investigation of Mommaerts and Seraidarian (16), according to which the enzymatic activity of myosin-ATPase *in vivo* cannot account for the liberation of inorganic phosphate during muscular activity.

The present study leads to a direct conclusion regarding the effect of ATP upon myosin through several equivocal arguments.

First, the effect of bivalent inorganic cations will be discussed. The enzymatic activity of myosin-ATPase is activated by Ca, and under almost all conditions inhibited by Mg. As shown in this paper, the viscosity response of actomyosin to which ATP is added is not inhibited, probably promoted by Mg, and is not promoted, probably inhibited by Ca. According to Szent-Györgyi (22, 23), the contraction of actomyosin threads is enhanced by Mg and inhibited by Ca. It is seen therefore that enzymatic splitting of ATP by myosin-ATPase, and the effect of ATP upon the physical behavior of myosin, are influenced by Mg and Ca in exactly the opposite way, a fact certainly not to be expected if the first of these two processes is the immediate cause of the second one.

Further arguments arise from the study of the effects of different substances related to ATP. Inosinetriphosphate is hydrolyzed by myosin-ATPase as readily as ATP, and the free energy effect of this reaction should be about the same as that of the splitting of ATP. Nevertheless the effect of ITP upon actomyosin is very much less pronounced than that of ATP, and is much more dependent on special conditions. Inorganic pyrophosphate and triphosphate, which are not hydrolyzed, affect actomyosin as ATP does, but at higher concentration and under more specialized conditions.

Finally the experiments described in section V show that after inactivation of the ATPase the viscosity response upon addition of ATP takes place undiminished. As shown by the absence of the recovery effect the enzymatic activity was destroyed completely.

These three independent groups of evidence allow us to conclude that the effect of ATP upon the physical behavior of myosin is entirely independent of the ATPase activity. The formation of a myosin-ATP compound through a topochemical reaction takes place as a result of which certain molecular proper-

ties of the myosin are changed. Whether myosin and ATPase are identical, or are two different proteins remains an important problem but has partly lost its significance with respect to the question of the energy transfer.

From these considerations it is not clear which rôle actin has. In the present experiments, actomyosin was used because under the circumstances studied myosin gives an observable effect only if it is combined with actin. Szent-Györgyi (23) seems to take the standpoint that changes in actomyosin are reflections of changes in the myosin moiety. According to him actin has no "active" rôle, it serves merely to keep the myosin in a proper condition. This deserves further study.

The physiological implications of the present results do not yet seem ripe for discussion. It has been shown that by combination with myosin, without hydrolysis, ATP induces physical changes in the myosin. According to the experiments with actomyosin threads such physical effects may reveal themselves as a contraction, if the conditions of the system are suitable. One arrives at the conclusion that the primary process consists of a combination between ATP and myosin; what follows this primary process depends on the conditions.

. The applicability of the results of these studies on myosin solutions to the physiological process of contraction is confirmed by the studies of Buchthal et al. (5–7) concerning the contractions evoked by ATP and other substances in intact muscle fibers. The gap between Buchthal's work and the present investigation with actomyosin solutions is bridged by the studies of Szent-Györgyi (23) on frozen and water-extracted muscle slices, and on actomyosin threads. It should be remembered that according to Caspersson and Thorell (8) in the resting muscle the ATP is not present in the contractile A bands.

Further consideration of the mechanism of contraction at this moment, when so many fundamental aspects of the process have not yet been sufficiently investigated, seems untimely.

SUMMARY

1. A study is made of the effect of adenosine triphosphate (ATP) upon the viscosity of solutions of actomyosin in 0.5 M KCl.

2. The observed effects are discussed in terms of an initial drop of the viscosity (viscosity response) and its subsequent slow reversal (recovery effect). The latter is ascribed to a decrease in the ATP concentration through enzymatic hydrolysis.

3. The recovery effect is inhibited by Mg, activated by Ca, in accordance with the effect of these ions on the activity of myosin-ATPase.

. 4. The viscosity response is not inhibited, probably promoted by Mg. It is not promoted, probably inhibited by Ca.

. 5. The viscosity response is induced not only by ATP, but to a certain ex-

tent also by inosinetriphosphate, inorganic triphosphate, and inorganic pyrophosphate, not by adenosine diphosphate or monophosphate.

6. The viscosity response could be obtained with enzymatically inactive myosin.

7. It is concluded that the effect of ATP upon myosin does not depend on its enzymatic hydrolysis.

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CORRECTIONS

In the article entitled "The quantic and statistical bases of visual excitation" (*J. Gen. Physiol.*, 1947–48, **31**, 269), in footnote 1, at the bottom of page 274, read, *The product* $k\,(k-1)\ldots$, *may be written* $\dfrac{k!}{(k-q)!}$.

On page 284, in equation (1) add, $t = m\tau; m = 1, 2, 3\ldots$.

I take this occasion to call attention to the fact that in 1944 van der Velden developed two equations, one of which described the variation of liminal energy with the flash time and the other this variation with the visual angle of the spot; both of them apply in the case of homogeneous populations of receptors (rods). These equations are very similar to mine, obtained in an entirely different manner and published nearly 4 years later. In fact, until very recently I was not aware of the equations of van der Velden, published in *Physica*, 1944, **11**, 179, because the papers published in that journal, and particularly those which are written in Dutch, generally become known to French physiologists as abstracts which are often incomplete.

Thus the priority of the theoretical basis of the empirical laws which are directly related to the two equations in question belongs entirely to van der Velden.

ERNEST L. M. BAUMGARDT

In Vol. 31, No. 3, January 20, 1948, page 261, in equation (1) for H_2 read H_1.

On page 262, in equation (5) for H read H_2.

On page 265, in the third line from the bottom of the page, for *4 c.mm.* read *5.24 c.mm.*

On the same page, in the second line from the bottom of the page, for *5 c.mm.* read *6.86 c.mm.*

GALLOXANTHIN, A CAROTENOID FROM THE CHICKEN RETINA*

BY GEORGE WALD

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A new carotenoid has been isolated from the chicken retina. It is proposed to call this *galloxanthin*, from *Gallus*, the generic name of the fowl, and the suffix *-xanthin*, which designates carotenoid alcohols or xanthophylls. This substance is interesting physiologically, as a new member of a series of carotenoid pigments which act as color filters for cone vision in the chicken (Wald and Zussman, 1938). It has an interest also for carotenoid chemistry, since its absorption spectrum lies in a wavelength region where—with one curious exception[1]—natural carotenoids have not previously been found.

Properties

Partition.—Galloxanthin is non-saponifiable, and displays no acidic properties. When partitioned between petrol ether and 90 per cent methanol, it goes almost entirely into the lower, alcohol layer (hypophasic). In partition between petrol ether and 80 per cent methanol, it is distributed about equally in both phases. This behavior is characteristic of xanthophylls.

Adsorption.—Galloxanthin is adsorbed very strongly from its solutions in petrol ether by calcium carbonate. This is another common xanthophyll property. The pigment when purified is adsorbed at the top of a column of calcium carbonate, and scarcely moves downward on prolonged washing with petrol ether. It does descend slowly on washing with pure benzene (C_6H_6).

In extracts of chicken retinas, galloxanthin is found mixed with astaxanthin, leaf xanthophylls, and a carotene. In the chromatogram on calcium carbonate, galloxanthin is adsorbed just below astaxanthin and above lutein. It cannot easily be separated from either pigment. All three carotenoids appear to form stereoisomeric sets (*cf.* Zechmeister, 1944), possibly as a result of extraction procedures, which overlap on the chromatogram.

[1] A carotenoid having an absorption spectrum in the same region as that of galloxanthin has recently been found in an artificially induced mutant of the yeast *Rhodotorula rubra* (pigment "B;" Bonner, Sandoval, Tang, and Zechmeister, 1946). Apart from its spectrum this pigment has very different properties from galloxanthin.

On saponifying such mixtures, astaxanthin is autoxidized to astacene. Galloxanthin can then be separated as a yellow band just below the red band of astacene, by developing the chromatogram on calcium carbonate with pure benzene.   An easier procedure is to chromatograph such a mixture out of petrol ether on powdered sugar; this holds astacene strongly, and allows galloxanthin to descend rapidly as a diffuse band, eventually to be collected in the filtrate.

Crystallization.—Attempts to crystallize this pigment have not yet succeeded. We have obtained granular precipitates repeatedly by bringing concentrated
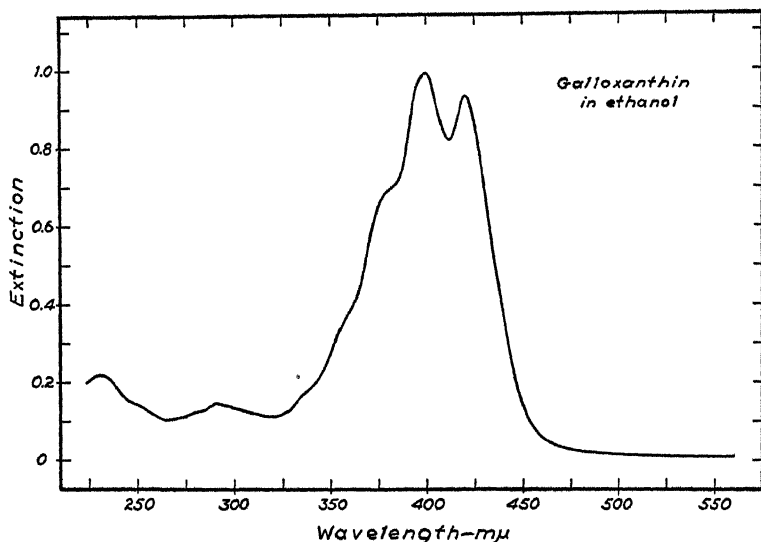
FIG. 1. Absorption spectrum of galloxanthin in solution in absolute ethanol.   The absorption is plotted in terms of the extinction, $\log I_0/I$, in which I_0 is the incident and I the transmitted intensity.

solutions in petrol ether to low temperatures or by adding water gradually to methanol or ethanol solutions in the warm and cold; but no frank crystals could be observed.   It should be recognized that all this work has been done with minimal quantities of the pigment, very much less than those with which one ordinarily pursues carotenoid chemistry.   This in itself has hindered many of the procedures.

Spectrum.—The spectrum of galloxanthin displays the three main bands characteristic of most carotenoids (Fig. 1).   In ethanol the absorption maxima lie at about 421, 400, and 378 mμ; in hexane at 422, 401, and (380 mμ); in chloroform at 427, 407, and (387 mμ); and in carbon disulfide at about 446 and 424 mμ.

In all our preparations the central band is most prominent, but the lateral

bands appear in various relations. In some preparations the longest wave-length band is reduced to a mere inflection, while the short wavelength band comes to a pronounced peak; in others, these relations are reversed. Fig. 2 shows examples of the types of spectrum observed. From their shapes and the procedures which yielded them, it seems probable that primarily they represent mixtures of *cis-trans* stereoisomers of galloxanthin in various pro-portions. No systematic study of this situation has yet been made, however, and other types of change may also be involved.
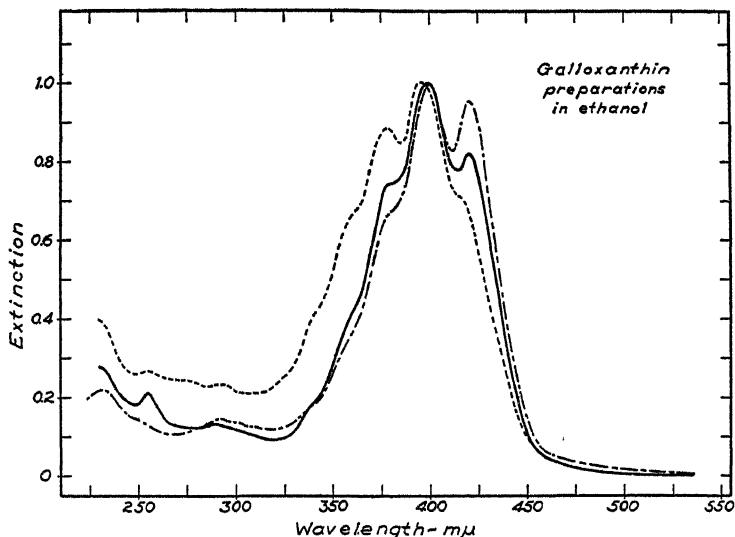
FIG. 2. Absorption spectra of three preparations of galloxanthin dissolved in absolute ethanol. These are selected to show the various forms in which the spectrum appears, probably representing primarily mixtures of *cis-trans* stereoisomers of gal-loxanthin in various proportions.

In addition to the main bands of galloxanthin, a small peak appears in some preparations at about 290 mμ (Figs. 1 and 2). The position of this band and its variability suggest that it is what Zechmeister has called a *"cis* peak," minimal in all-*trans* carotenoids, and prominent in those *cis* forms in which the molecule is appreciably bent. According to Zechmeister the *cis* peak almost invariably lies at 142 $\pm$ 2 mμ below the longest wavelength maxi-mum of the all-*trans* form in hexane. In galloxanthin this would place it at about 280 mμ, slightly below the observed position. There is also in some preparations the suggestion of a small band at about 255 mμ, and one at about 230 mμ.

Galloxanthin in ultraviolet light has not been observed to fluoresce either in solution or when concentrated by adsorption on the chromatogram.

Antimony Chloride Reaction.—Galloxanthin yields the deep blue color characteristic of many carotenoids when mixed with saturated antimony trichloride in chloroform. The color develops immediately, then slowly fades as is usual in this test. The spectrum of the transient blue product, recorded within 1 minute in the Hardy photoelectric spectrophotometer, rises from about
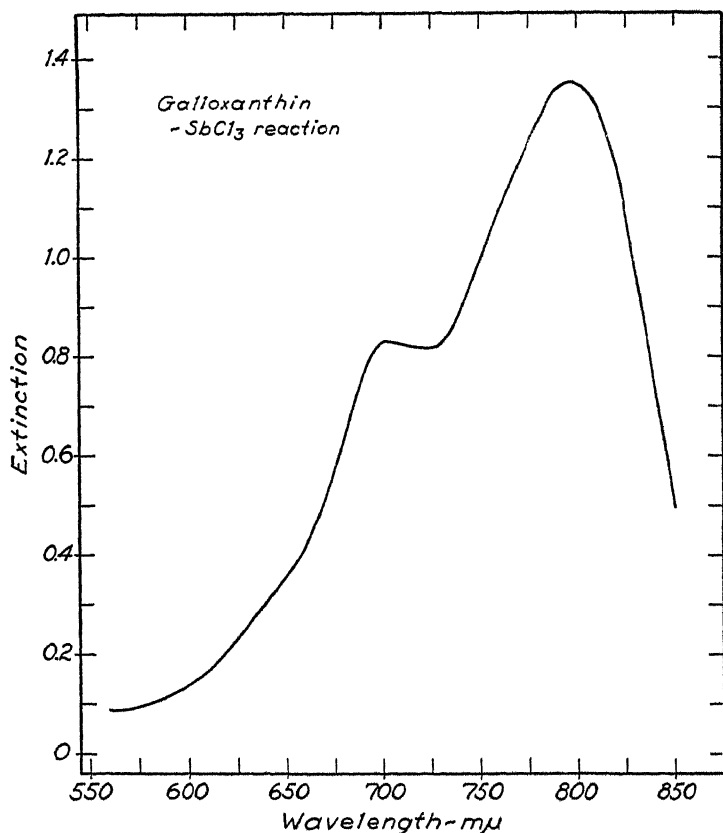
Fig. 3. Absorption spectrum of the blue product obtained by mixing a preparation of galloxanthin with antimony trichloride. The same concentration of galloxanthin in simple solution in chloroform would have an extinction at 405 mμ of 1.0.

560 mμ to an inflection at about 700 mμ, as though a maximum lay just beyond. Unfortunately this instrument does not record longer wavelengths.

We have attempted to measure the antimony chloride spectrum with the Beckman spectrophotometer, which is too slow to make this kind of measurement conveniently. Working as rapidly as possible, however, we have pieced out the spectrum in short, overlapping ranges, completing each series of measurements within 1.5 minutes after mixing the reagents. The result

with one preparation of galloxanthin is shown in Fig. 3; it is reasonably typical of all our preparations.

In the antimony chloride reaction a main absorption band is always found at 785 to 795 mμ. In various preparations the height of this band remains fairly proportional to the height of the direct spectrum. Thus the extinction in the antimony chloride test at about 790 mμ is on the average 1.35 times (range 1.17 to 1.60) as great as the extinction at 405 mμ of the same concentration of galloxanthin in chloroform. This information has been incorporated in Fig. 3, which shows the spectrum of the antimony chloride product of a preparation of galloxanthin which, in the same concentration in chloroform and in the same depth of layer, would have had an extinction at 405 mμ of 1.0.

A secondary maximum in the antimony chloride test is found at about 710 mμ. The relations between this and the main maximum vary greatly from one preparation to another. It is probable that the absorption in this region is due either to some minor modification of galloxanthin or to another type of contaminant.

DISCUSSION

Two properties of galloxanthin deserve special attention, since they have implications for its structure: (a) the location of its absorption spectrum; and (b) its extraordinary adsorbability.

The position of the main absorption spectrum of a carotenoid or synthetic polyene is determined primarily by the length of its conjugated system of double bonds (Radulescu and Barbulescu, 1931; von Euler, Karrer, Klussmann, and Morf, 1932; Hausser, Kuhn, and Smakula, 1935). Among all such substances which have been investigated, only one of known structure absorbs in the same spectral region as galloxanthin. This is the carotenoid derivative, dihydrobixin (von Euler, Karrer, Klussmann, and Morf, 1932). It possesses eight conjugated double bonds; and also two carboxyl groups in non-conjugated positions which probably have little effect upon its visible spectrum. The spectrum of dihydrobixin resembles greatly that of galloxanthin in both shape and position. It seems probable from this comparison and from the location of its spectrum relative to other carotenoids, that galloxanthin possesses eight conjugated double bonds.

It may be recalled that another retinal carotenoid, retinene₂, lies close in the spectrum to galloxanthin. Its single absorption band is maximal at about 405 mμ in chloroform (Wald, 1938–39). It happens that retinene spectra, however, are displaced extraordinarily far toward longer wavelengths in chloroform solution, as compared with such homopolar solvents as hexane. The absorption maximum of retinene₂ in hexane lies at about 374 mμ, far below the position of the main galloxanthin band in this solvent. These pigments, therefore, share only a limited coincidence in spectral location.

Other structural considerations apart, the adsorbability of carotenoids also varies with the length of conjugated system.   One should, for this reason, expect that galloxanthin, with a relatively short conjugated system, might be weakly adsorbed.   It has instead an extraordinarily high adsorption, stronger than that of lutein with eleven conjugated double bonds and two hydroxyl groups; and only slightly weaker than that of astaxanthin, with eleven conjugated double bonds, two keto groups probably conjugated with the polyene chain, and two hydroxyls.   It is clear that the structure of galloxanthin must include other features making for strong adsorption, which more than compensate for the shortness of its conjugated system.   It may for example possess more than two hydroxyl groups.   There are other, subtler possibilities; witness the observation that phytofluene, apparently a hydrocarbon containing only five conjugated double bonds, displays a similarly "abnormal" adsorbability (Zechmeister and Sandoval, 1946).

Finally, something may be said of the physiological significance of galloxanthin.   It occurs free in the retina, and can be extracted from the desiccated tissue by merely shaking with petrol ether.   It probably is to be ranged therefore with the group of carotenoids found in free solution in the oil droplets of the retinal cones.

These pigments are so placed as to act as color filters for the individual cones.   The effectiveness of galloxanthin as a filter may be judged from the fact that it is isolable from the chicken retina in such amount that if spread evenly over the whole retinal surface it would have a maximal absorption of about 50 per cent.   Since its extraction and isolation necessarily entail losses, and since it probably is not distributed homogeneously over the retina, its density *in situ* may be considerably greater.

The other filter pigments of the chicken retina—astaxanthin, lutein, and the retinal carotene—absorb at considerably longer wavelengths than galloxanthin.   Indeed their absorptions fall off in the very region of the violet in which the galloxanthin absorption becomes maximal.   Galloxanthin is therefore in position to complement the filtering action of the other pigments; it keeps high the absorption in the violet and near ultraviolet, where it would otherwise be small.

What is the significance of such absorption?   It is a reasonable view, as has been suggested many times, that the filter pigments of the chicken retina are used in color discrimination, much as three-filter systems are employed in color photography.   This is therefore one possibility—that galloxanthin acts as an auxiliary to the other pigments in color differentiation.   There is another function, however, for which it is more specifically fitted.   It is reasonably certain that the chicken eye, like the human eye, possesses a large chromatic aberration.   That is, when the eye is in focus for the middle region of the visible spectrum, it is highly myopic and hence considerably out of focus for short wavelengths (Wald and Griffin, 1947).   Potentially this should

result in a considerable blurring of the retinal image. In the human eye, however, this effect is avoided by excluding most of the violet and virtually all the ultraviolet through the filtering action of the yellow lens. In the chicken the lens is not visibly colored; and here it is possible that the same removal of low wavelengths is accomplished by galloxanthin.

SUMMARY

A new carotenoid has been isolated from the chicken retina for which the name *galloxanthin* is proposed. This substance has the properties of a hydroxy carotenoid or xanthophyll. It has not yet been crystallized. On a chromatogram of calcium carbonate it is adsorbed just below astaxanthin and above lutein.

The absorption spectrum of galloxanthin lies in a region where natural carotenoids have not ordinarily been found. Its main, central absorption band falls at about 400 mμ. The position of its spectrum suggests a conjugated system of eight double bonds. This relatively short polyene structure must be reconciled with very strong adsorption affinities.

With antimony trichloride, galloxanthin yields a deep blue product, possessing a main absorption band at 785 to 795 mμ, and a secondary maximum at about 710 mμ which may not be due to galloxanthin itself.

Galloxanthin appears to be one of the carotenoid filter pigments associated with cone vision in the chicken. It may act as an auxiliary to the other filter pigments in differentiating colors; or its primary function may be to exclude violet and near ultraviolet radiations for which the eye has a large chromatic aberration.

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THE EFFECTS OF LOW TEMPERATURE ON FERTILIZED RABBIT OVA IN VITRO, AND THE NORMAL DEVELOPMENT OF OVA KEPT AT LOW TEMPERATURE FOR SEVERAL DAYS

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PLATES 8 TO 10

The life span of germ cells is limited. The viability of mature rabbit spermatozoa in the scrotal epididymis is preserved up to 38 days, but for only 30 hours in the female tract following ejaculation (Hammond and Asdell, 1926). Rabbit sperms can be kept *in vitro* for 96 to 168 hours at 10° or 15°C. without complete loss of fertilizing capacity (Hammond, 1930; Walton, 1930). At body temperature, however, the maximal survival time *in vitro* is only 13 hours (Walton, 1930). The relatively short life of mature ovarian follicles in the rabbit which appear and disappear within the space of a few days (Smelser, Walton, and Whetham, 1934), indicates that the life span of mature ova in the absence of ovulation is short. After ovulation, rabbit ova are capable of fertilization *in vivo* only for 6 hours, as determined by Hammond (1934), or 9 hours, as estimated by Pincus (1936).

Following the discovery by Ivanov (1907, 1926) that low temperature storage prolongs the viability of mammalian spermatozoa, the effect of low temperatures on spermatozoa has been extensively investigated in recent years, due both to academic interest and to the practical value of artificial insemination in animal husbandry (*cf.* Anderson, 1945).

The effect of low temperature on the ova of marine organisms has been investigated in connection with the temperature coefficient of cell division (*cf.* Gray, 1931). That the fertilized ova of *Ascaris equorum* can be kept at 0°C. for 2 months has been reported by Beams and King (1940). The development of fish at various temperatures (9–21°C.) was studied by Worley (1933); triploid newts were obtained by exposing the fertilized ova to 0–3°C. (Fankhauser and Griffiths, 1939; Griffiths, 1941). The activation of unfertilized rabbit ova by means of high and low temperatures *in vitro*, as well as *in vivo*, has been reported by Pincus and his associates (Pincus and Enzmann, 1936; Pincus, 1939 *a*; 1939 *b*; Pincus and Shapiro, 1940). However, the effect of low temperatures on fertilized rabbit ova *in vitro* has not yet been investigated.

In view of the fact that the life span of ejaculated spermatozoa is shorter at body temperature *in vitro* as well as *in vivo* than at low temperature *in vitro*, and in view of the fact that delayed implantation is fairly common in certain

mammals, Mustelidae and Muridae (*cf.* Asdell, 1946), it was thought possible that the fertilized ovum might be kept *in vitro* at low temperature for a period of time without loss of its capacity for normal development.

This paper reports a series of experiments dealing with: (1) the rate of cooling and the subsequent viability of fertilized rabbit ova; (2) the optimal temperature for the preservation of the viability of fertilized rabbit ova; (3) the normal development *in vivo* of rabbit ova transplanted after several days' storage at low temperatures *in vitro*; and (4) related observations including (*a*) fertilization of superovulated ova, (*b*) compatibility of serum and ova from different animals, (*c*) viability of ova fertilized by spermatozoa from different animals.

Materials and Methods

Superovulation of Rabbits.—Adult or 6 to 9 months old non-pregnant rabbits of mixed breeding were injected 6 times subcutaneously with sheep pituitary extract in order to stimulate the growth of a large number of follicles (Pincus, 1940). The rabbits were mated or artificially inseminated (*cf.* Chang, 1946) just before the final intravenous injection of the same pituitary extract to induce ovulation. About 20 minutes before sacrifice, rabbits were bled by heart puncture to obtain serum. They were killed 24 to 24½ hours after insemination. Usually four does were killed at a time. The ova were flushed out from the oviducts with pure rabbit serum. About 100 ova could be recovered by these procedures when the total subcutaneous dose of pituitary extract was 57.6 r.u. and the ovulating dose 12.8 r.u.

Serum as Storage and Culture Medium.—The serum was obtained by centrifugation of whole blood for 20 to 30 minutes at 2000 R.P.M. Homologous sera as well as hetero-logous sera were used for flushing, storage, and culture. The origin of sera used in each case was recorded. Fresh serum, or serum not more than 24 hours old and kept in the refrigerator, was used throughout this investigation.

Manipulation of Ova.—The methods for the manipulation of ova were those described by Pincus (1936). The procedures were carried out inside a glass cabinet in the culture room kept at about 30°C. After the ova had been flushed into a watch glass placed in a Petri dish containing a piece of moistened filter paper, they were examined under a binocular dissecting microscope which was mounted in the glass cabinet. Most of the ova thus recovered were in the 2-blastomere stage. Occasionally, unfertilized, fertilized, but not cleaved ova, and 3-4-blastomere ova were obtained. It was quite easy to distinguish the unfertilized from the fertilized but uncleaved ovum. The latter has one big or two small nuclei resembling small light shallow discs. The former is not clear, is without obvious nuclei (Fig. 1). A group of about 5 to 20 fertilized ova was picked up with a pipette and dropped into a very small pyrex flask filled to the neck with 1 ml. of pure serum. These flasks were closed with a small rubber bulb and tied with thread prior to the treatment at low temperature.

Sterile conditions and aseptic precautions were maintained as strictly as possible in the operating room, culture room, and culture cabinet.

Temperature Control.—A thermos flask filled with ice and kept in the cold room at 1°C. was used as a container for the storage of ova at 0°C.; ordinary serological

baths kept in the same room were set up at 5°, 10°, or 15°C. for the storage of ova at these temperatures.

In order to control the rate of cooling, the small flasks containing ova were first dipped in a 500 ml. beaker containing 200 ml. of water at 25°C. The beaker was transferred to a refrigerator at 4°C. The temperature of water in the beaker fell to 15°C. in about 1 hour, to 10°C. in 2 hours, and to 5°C. in 4 hours. This procedure was adopted to slow down the rate of cooling. If cooling to 0°C. was required, the flasks were kept at 5°C. for 1 hour and then transferred to ice.

Culture of Ova.—After the storage of ova at low temperatures for various lengths of time, the serum containing ova was sucked up with a pipette and dropped into a watch glass placed inside a Petri dish. It was then examined under the dissecting microscope. The number of ova and the condition of the ova were recorded. The ova were then transferred to a 10 ml. Carrel culture flask containing 4 ml. of serum. These culture flasks were attached to a shaker and incubated at 38°C. (Shapiro, 1939). After 20 to 24 hours of culture, the ova were taken out and examined to determine their viability.

Criteria of Viability.—In determining the viability, survival, and the death of the ova after incubation, the following methods and criteria were adopted. The ova were first examined with a magnification of 36, then with a magnification of 72. If the serum was not clear enough, the ova were transferred to saline for detailed observation. The condition of the ova was recorded in the protocols. In final treatment of the data, however, the condition of the ova was classified as follows (Figs. 4 to 6):

1. Dead ova:
    1 blastomere: includes uncleaved or fragmented uncleaved fertilized ova.
    2–3 blastomere: includes regular 2- or 3-blastomere ova; irregular or fragmented 2- or 3-blastomere ova.
2. Abnormally cleaved ova (retarded cleavage and irregular cleavage).
    4–5 blastomere: 4- or 5-blastomere ova, in which the blastomeres were regular, irregular, or fragmented.
    6–8 blastomere: Ova with 6, 7, or 8 regular, irregular, or fragmented blastomeres; also ova with 10 or more irregular blastomeres.
3. Viable ova.
    12–16 blastomere: regular 12–16 or more blastomere ova. Only ova belonging to this category were considered as ova viable after treatment; the percentage in each group was calculated.

Histological Techniques.—Ova, after different treatments, in addition to examination under the dissecting microscope, were fixed in a clot of serum (Pincus, 1939 a) with Bouin's solution, sectioned at 10 micra, and stained with Harris' hematoxylin for microscopic examination.

Transplantation of Ova (Pincus, 1936).—The recipient doe was injected with pituitary extract intravenously 18 to 24 hours previously in order to induce ovulation. Veterinary nembutal was injected intravenously and ether was administered later. The viable ova after culture were sucked into a pipette. The tip of the pipette was inserted into the infundibulum of the oviduct and the ova were evacuated by squeezing the rubber bulb of the pipette. Four to eleven ova were transplanted into each recipient doe. Aseptic precautions were maintained throughout.

1. The Rate of Cooling and the Survival and Viability of Fertilized Ova

It was demonstrated by Chang and Walton (1940) that sudden cooling has a harmful effect ("temperature shock") on the subsequent viability of ram spermatozoa. These investigators also found that temperature shock can be prevented by cooling the ram spermatozoa gradually, in which case the subsequent viability of spermatozoa is benefited by acclimatization. Experiments were undertaken to determine whether ova are also subject to the harmful effect of sudden cooling, and whether slow cooling, "acclimatization," is beneficial to subsequent survival.

TABLE I

The Effect of Slow Cooling and Rapid Cooling on the Subsequent Survival of Fertilized Rabbit Ova

Storage temperature	Method of cooling	No. of ova	Ova dead	Ova survived		
				Abnormal cleavage	Normal cleavage	Total
°C.			per cent	per cent	per cent	per cent
0	Slow	41	24.4	12.2	63.4	75.6
	Rapid	45	33.3	40.0	26.7	66.7
	Difference....		−8.9	−27.8	+36.7	+8.9
5	Slow	47	14.9	10.6	74.3	84.9
	Rapid	48	10.4	41.6	47.9	89.5
	Difference.....		+4.5	−31.0	+26.4	−4.6
10	Slow	31	0	19.3	80.6	99.9
	Rapid	34	2.9	20.6	76.5	97.1
	Difference.....		−2.9	−1.3	+4.1	+2.8

Ova of the same rabbit were divided into two groups and stored in two small flasks containing aliquots of the same serum. One flask was immediately dipped into 10°, 5°, or 0°C. water (rapid cooling). The other was cooled from 25–10°C. in 2 hours, or cooled to 5°C. in 4 hours, or to 5°C. in 4 hours and acclimatized at 5°C. for another hour before dipping into ice water (slow cooling). After storage at the respective temperatures for 24 hours, the ova were transferred to a Carrel flask and incubated for 24 hours at 38°C. They were then examined. Table I shows the composite results of six tests for each treatment.

It is evident from the data in Table I that rapid cooling to 0°C. was harmful to the subsequent viability of fertilized ova as compared with slow cooling to 0°C. The difference between six tests for each treatment is statistically significant ($t = 2.63$, $p < 0.05 > 0.02$). Although the difference between slow cooling and rapid cooling to 5°C. was not statistically significant ($t = 1.30$, $p < 0.2 > 0.03$), perhaps due to the small number of tests, considerable

difference is shown. There was, however, not much difference between those ova cooled slowly or rapidly to 10°C. This experiment indicates that ova, like spermatozoa, are subject to the harmful effect of rapid cooling "temperature shock," and that the rate of cooling plays an important part in the subsequent viability of ova when the storage temperature is low (5–0°C.).

It is interesting to note, from Table I, that there was no difference in the percentage of dead and surviving ova (including normal and abnormal cleavage) after slow or rapid cooling to 0° or to 5°C. But there was a large increase in the percentage of abnormal cleavages when ova were cooled rapidly. These facts show that rapid cooling as compared with slow cooling did not really kill the ova but did impair their normal functions. In other words, slow cooling or acclimatization did not prevent the death of ova at low temperatures, but did prevent the deterioration of survivors during the process of cooling.

2. The Optimal Temperature for the Storage of Fertilized Rabbit Ova

For the determination of the optimal storage temperature of fertilized ova, the ova were cooled slowly to 0° or to 5°C. according to the standard technique. Since there was no difference in the viability of ova following slow cooling or rapid cooling to 10°C., the ova were transferred directly from 25°C. to a water bath at 10°C. for storage. Groups of ova in small flasks were placed in a bath at 15°C. or kept in the room (22-24°C.). After storage for 1 to 7 days the ova were taken from the small storage flasks, examined, and cultured.

It should be noted here that no one of the ova cleaved during the storage at 0–15°C., no matter how long they were kept there. However, some ova did cleave once at room temperature.

According to Gregory (1930) and Pincus (1936), most ova are in a 16–32 cell stage *in vivo* 48 hours after mating. Since the ova used in this experiment were recovered 24 to 25 hours after insemination, and since the ova did not cleave during storage, they should be at the 16–32 cell stage after 24 hours' culture if *in vitro* culture has no adverse effect on the rate of cleavage. It is true that in some cultures, ova did cleave to the 32 cell stage (Figs. 9 and 10), but ova with 12 or more blastomeres were also considered as normally cleaved (viable ova) (Figs. 7 and 8).

The complete results derived from 101 tests are presented in Table II. In each determination, 3 to 8 tests were performed, 5 to 20 ova were examined in each test. Text-fig. 1 shows graphically the viability of ova after storage at different temperatures for various lengths of time. It is obvious that 10°C. is the optimal temperature for the storage of rabbit ova, since only at this temperature viable ova were obtained after 144 hours' storage.

A temperature of 0°C. manifested a severe effect at the beginning (24 hours' storage), while a temperature of 15°C. manifested a beneficial effect at the same time, but at either temperature the decrease of viability followed a rather similar course thereafter. At 10°C., there was no severe harm done at the

beginning, but there was also protection against delayed deleterious changes. Thus, 10°C. appears to be the optimal temperature. Room temperature is

TABLE II

The Viability of Fertilized Rabbit Ova Cultured at 38°C. after Storage at Different Temperatures for Various Lengths of Time

Storage time	Storage temperature	No. of ova	Ova dead after storage	Ova survived after storage		
				Abnormal cleavage	Normal cleavage	Total
hrs.	*°C.*		*per cent*	*per cent*	*per cent*	*per cent*
24	0	41	24.4	12.2	63.4	75.6
	5	60	11.6	10.0	78.3	88.3
	10	45	0	17.7	82.2	100.0
	15	55	5.5	9.1	85.5	94.6
	24	43	6.9	55.8	37.2	93.0
48	0	13	7.7	30.8	61.5	92.3*
	5	68	11.7	22.0	66.2	88.2
	10	—	—	—	—	—
	15	54	16.6	31.4	51.9	83.3
	24	30	33.3	56.7	10.0	66.7
72–80	0	54	33.3	18.5	48.1	66.6
	5	120	33.3	12.5	54.2	66.7
	10	51	17.6	23.5	58.8	82.3
	15	51	27.5	29.4	43.1	72.5
	24	8	75.0	25.0	0	25.0
96–102	0	60	46.7	35.0	18.3	53.3
	5	80	47.5	17.5	35.0	52.5
	10	39	23.0	25.6	51.3	76.9
	15	61	59.0	29.5	11.5	41.0
120	0	39	92.3	7.7	0	7.7
	5	80	63.8	30.0	6.3	36.3
	10	52	25.0	38.5	36.5	75.0
	15	31	83.8	16.1	0	16.1
144	10	55	25.5	50.9	23.6	74.5
168	10	35	80	20	0	20

* Small number of ova tested, not shown in figures.

not at all favorable for the storage of ova. Perhaps this temperature is not low enough to inhibit the physiological activity of the living tissue, with the result that some sort of exhaustion of vital energy may occur in a short time. It is also possible that deleterious changes occur at this temperature.
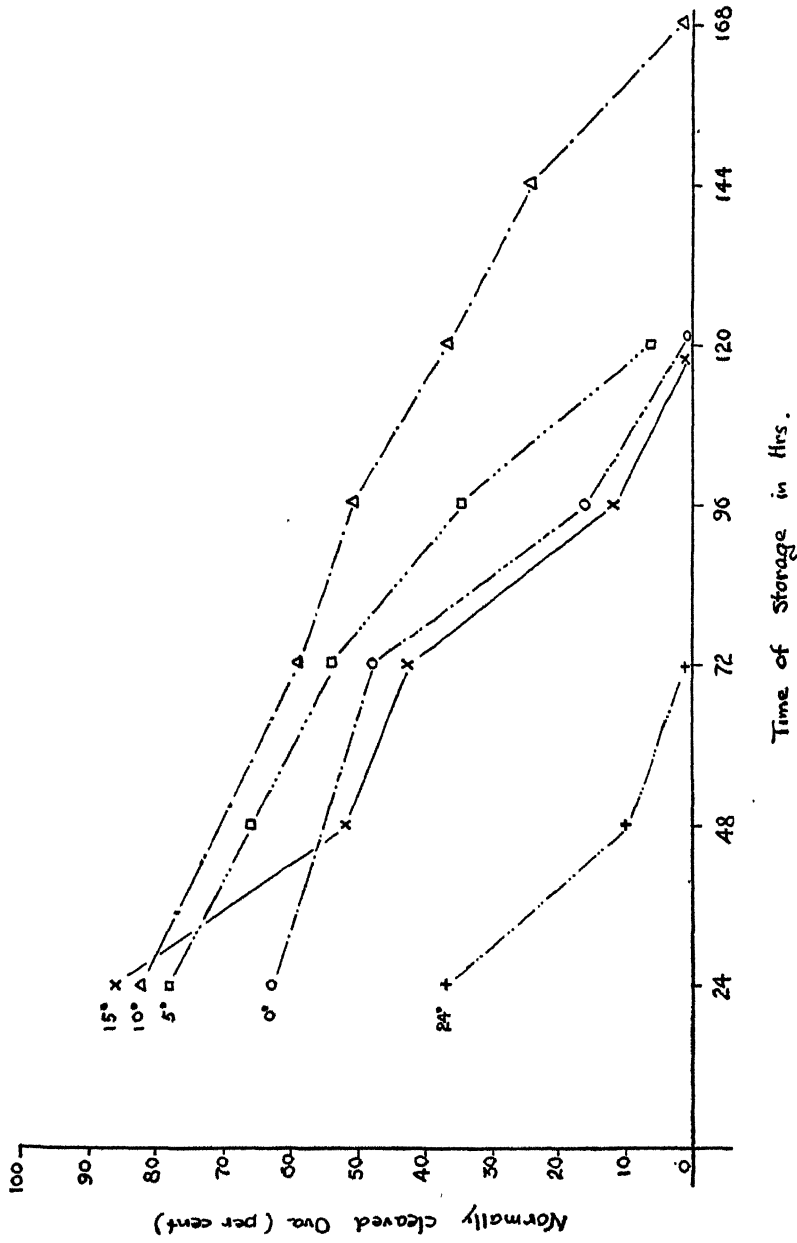
TEXT-FIG. 1. Effect of low temperatures on the viability of fertilized rabbit ova.

The number of normally cleaved ova present after varying periods of storage follows a more or less linear curve (Text-fig. 1), no matter what temperature was employed, with the possible exception of storage at 0°C. Thus, the percentage of viable ova decreased proportionally as time elapsed, though the rate of decrease was not the same at different temperatures.

The time factor is involved in the evaluation of a favorable temperature. For a storage of 24 hours, there was practically no difference between 5° and 15°C. For a storage of 72 hours, the difference between 10° and 5°C. may not have been significant, but there was a difference between 10° and 15°C. After 72 hours, there was a difference at all temperatures employed. It seems, therefore, that the differential effect of various temperatures is exerted only after a certain critical time of storage; *i.e.*, after 72 hours.

It is obvious that any particular temperature has its own characteristic effect on the proportion of dead ova and the production of abnormally cleaved ova as shown in Text-figs. 2 to 6. At the most favorable temperature (10°C.), the percentage of dead ova kept at a low level for a longer time then increased very fast on the last day. At a less favorable temperature (0°C. or 5°C.), it remained at a low level for a relatively short time, but increased steadily thereafter. At an unfavorable temperature (15° or 22 to 24°C.), it increased steadily and rapidly from beginning to end. The percentage of abnormally cleaved ova followed neither the course of normally cleaved ova nor the course of dead ova. At a low temperature (0–10°C.), it stayed at low level for 3 to 4 days, then increased steadily just before or during the time when the percentage of dead ova increased, thereafter it fell at the end. At a high temperature, such as at 15°C., it increased at the beginning; at a still higher temperature (22–24°C.), it remained at a high level at the beginning and then fell off rapidly. The occurrence of a high percentage of abnormally cleaved ova is more or less coincident with the increasing rate of death in ova, which shows that the disintegration of normal physiological functions precedes the death.

Experiments were performed to determine whether or not the fertilized ova could stand vitrification. Ova kept either in a capillary glass tube, or in small flasks, or in a test tube (1 cm. diameter) with pure serum or serum containing 0.5 to 1 per cent of fructose to dehydrate the ova slightly, were dipped into solid carbon dioxide in acetone at a temperature of −65°C. for 30 minutes. The ova were cultured according to the standard technique. Not one of the ova, however, survived this treatment.

3. The Normal Development of Young Following Transplantation of Ova Stored at Low Temperatures for Several Days and Cultured at 38°C. for 24 Hours

The results of this experiment are presented in Table III. After transplantation of ova stored at 0°C. for 78 to 102 hours, four litters of one to two young each were obtained. Four litters (two to seven young) were delivered
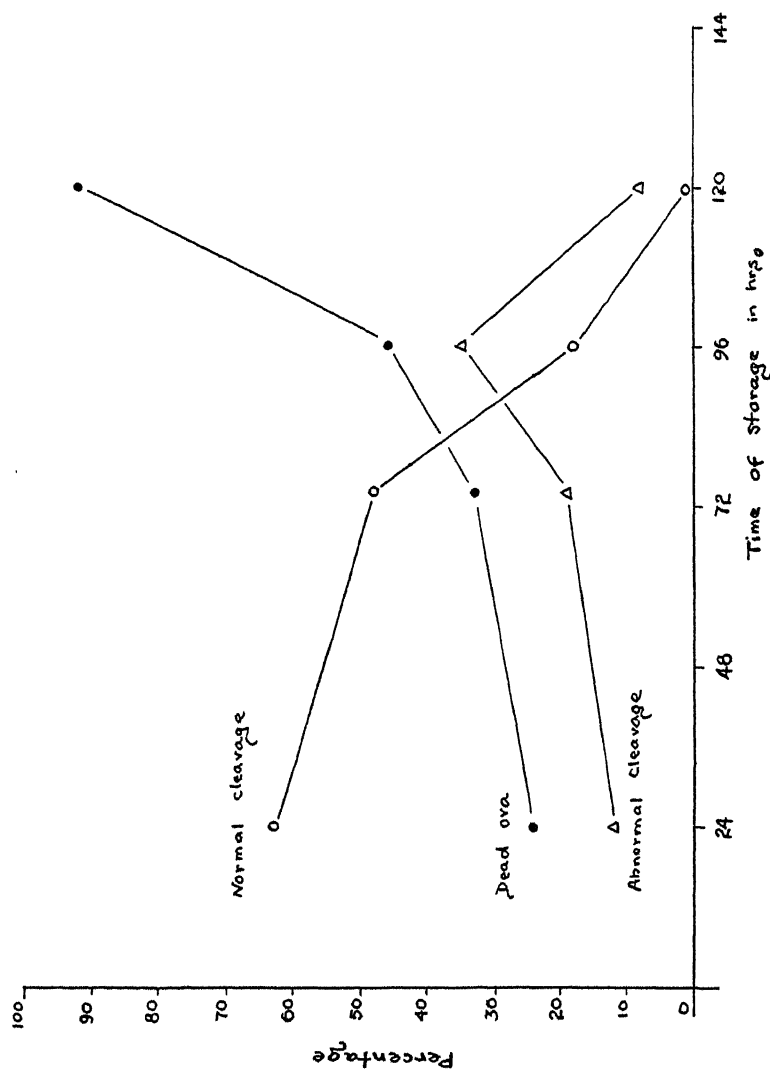
TEXT-FIG. 2. Normally cleaved, abnormally cleaved, and dead ova after storage at 0°C.
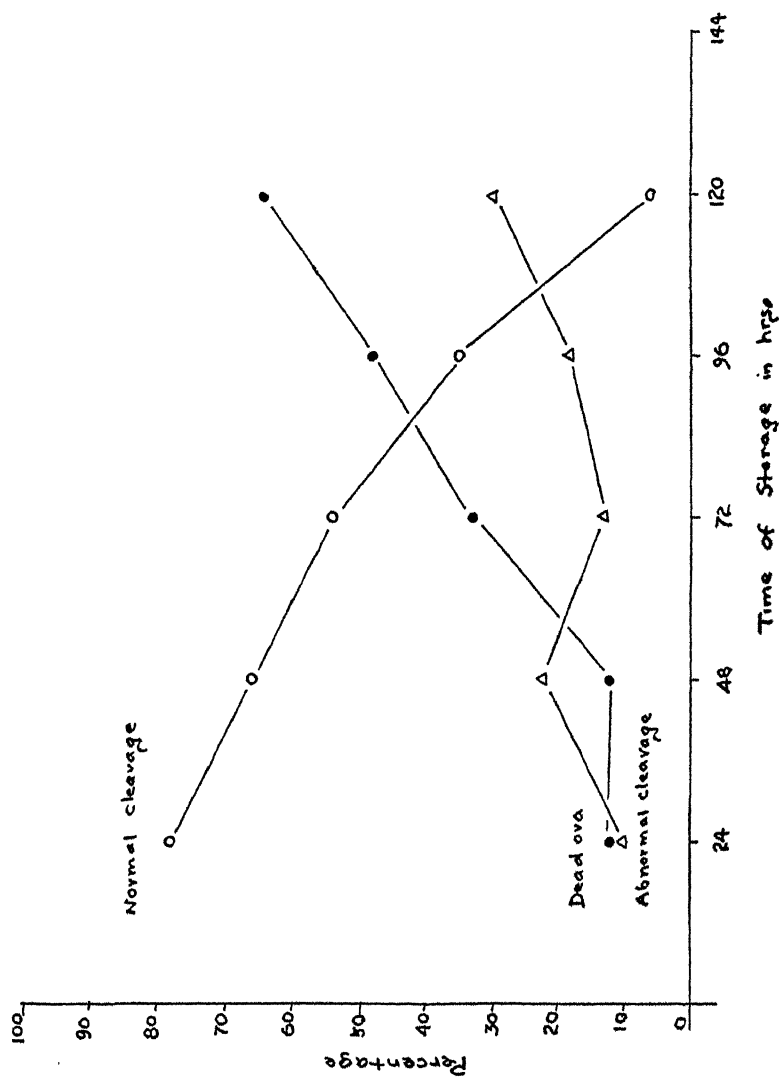
TEXT-FIG. 3. Normally cleaved, abnormally cleaved, and dead ova after storage at 5°C.
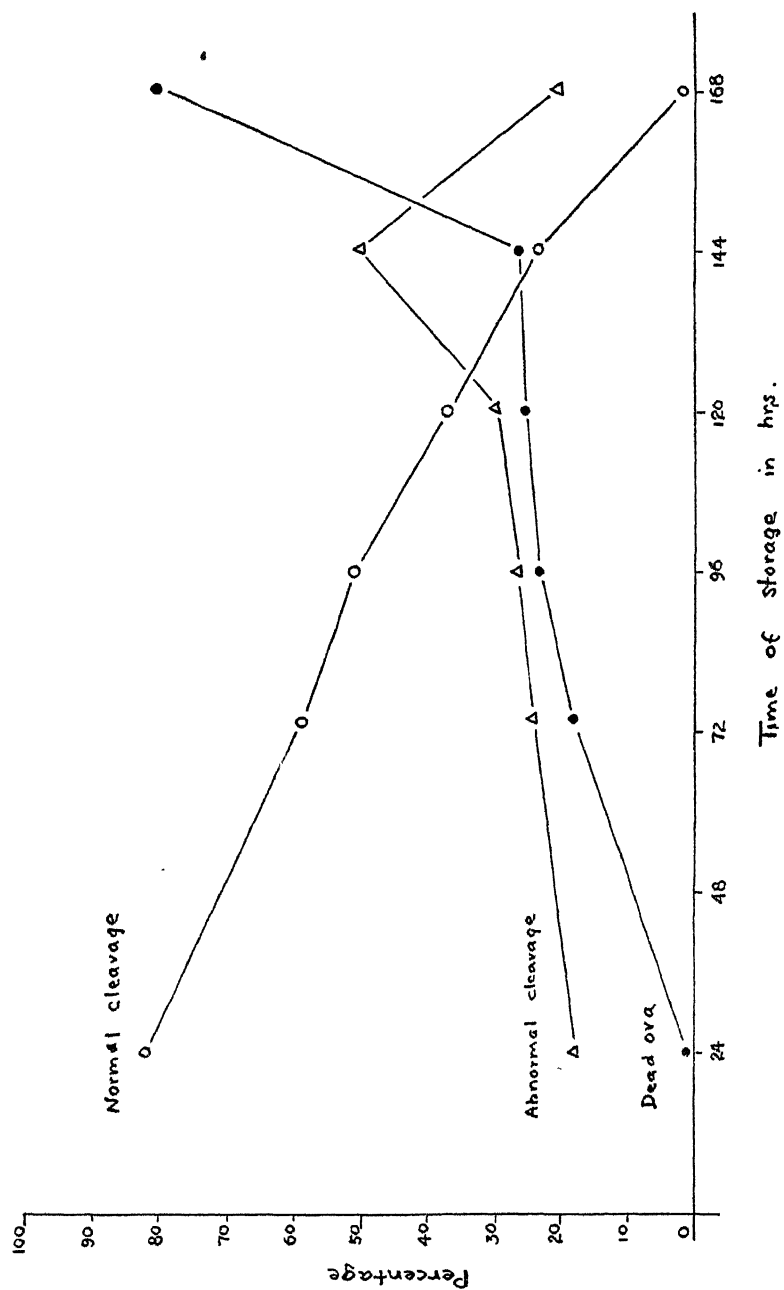
TEXT-FIG. 4. Normally cleaved, abnormally cleaved, and dead ova after storage at 10°C.

from eight recipient does following transplantation of ova stored at 5°C. for 48 to 80 hours. Four litters of one to four young were delivered from five recipient does after transplantation of ova stored at 10°C. for 77 to 101 hours. One litter of one young was obtained from two recipient does after trans-
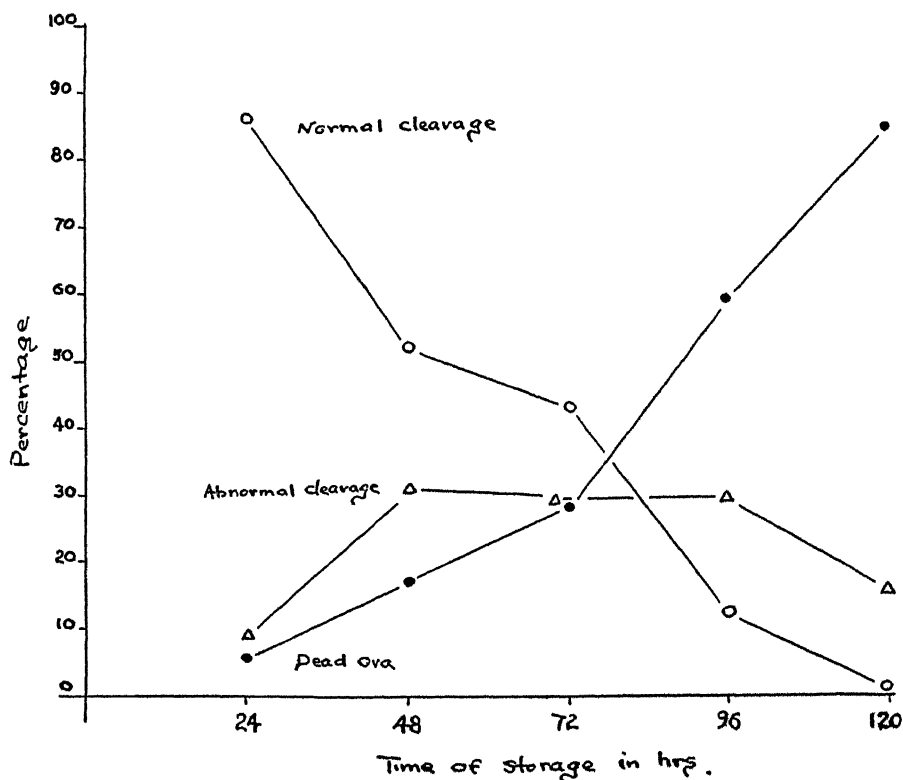
TEXT-FIG. 5. Normally cleaved, abnormally cleaved, and dead ova after storage at 15°C.

plantation of ova stored at 15°C. for 72 to 96 hours. All the young born were healthy, normal, and genetically true to their real parents (Fig. 20).

Finally, four does (Nos. 3-45, 2-80, 3-63, and 3-77) were transplanted with normally cleaved ova after culture following storage at 10°C. for 120 to 144 hours. None of them became pregnant. One doe (No. 3-64), who received four normally cleaved ova following storage of 101 hours at 5°C., did not become pregnant. One doe (No. 2-79), who received six ova which had shown retarded cleavage at 38°C. following 120 hours' storage at 5°C., gave birth to one small abnormal offspring at full term. Considering the

small litter size in cases of successful transplantation and the failure of pregnancy after longer times of ova storage, it is evident that the ova which cleaved normally in culture are not necessarily sufficiently viable for full development.

It should be noted here that the sex ratio of young (19 males *vs.* 13 females) has not been disturbed after low temperature treatment of the fertilized ova.
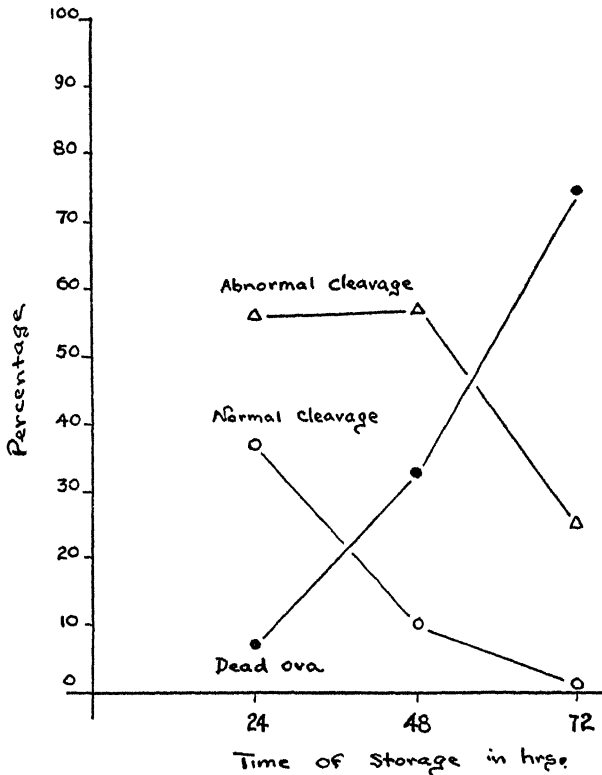
TEXT-FIG. 6. Normally cleaved, abnormally cleaved, and dead ova after storage at 24°C.

Table IV, derived from Table III, shows the probability of normal development following transplantation of ova stored at low temperature and cultured; *i.e.*, the number of ova transplanted and the number of normal young obtained. Though the number of cases is too small for any definite conclusions, the table indicates (1) that the ability of ova to develop into normal animals was better when the ova were stored at the low temperatures, 0–10°C., and (2) that the probability of development of those ova normally cleaved in culture after 2 to 4 days' storage was 1 out of 4.

TABLE III

Normal Development of Young Rabbits Following Transplantation of Ova Kept at Low Temperatures for 2 to 4 Days and Cultured at 38° for 1 Day

Storage temperature	Recipient doe	Ova from	Fertilized by sperm of	Time of Storage	No. of ova trans. L	No. of ova trans. R	Length of pregnancy	Young ♂	Young ♀	Total	Remarks
°C.							days				
0	Alb.	Chin.	Chin.	78	5	4	34	1 Chin.	1 Chin.	2	
	Chin.	Chin.	Chin.	78	5	4	34		1 Chin.	1	
	Chin.	White	Alb.	96	6	0		2 normal fetuses 1 degenerated fetus		2	Recipient mother died
	Red	Alb.	Alb.	102	3	2	31	1 Alb.	1 Alb.	2	
5	Bla.	Alb.	Alb.	48	4	3	No pregnancy				
	Engh.	Alb.	Alb.	48	3	3	No pregnancy				
	Bla.	White	AnEng.	48	5	5	32	1 White 1 Chin. 1 Engh.	1 White 1 Chin.	5	
	Bla.	White	AnEng.	48	5	5	30	3 White 1 Alb.	1 Engh. 1 White 1 Alb.	7	
	Bla.	Alb.	Alb.	80	4	4	No pregnancy				
	Red	Chin.	Chin.	80	5	5	35	1 Chin.	1 Chin.	2	
	Red	Chin.	Chin.	80	5	6	No pregnancy				
	Bla.	Alb.	AnEng.	80	5	2	31	2 White	1 White	3	
	Alb.	Alb.	AnEng.	101	4	0	No pregnancy				
	Bla.	Alb.	AnEng.	120	6*	0	32	A small abnormal			
10	Bla.	Alb.	AnEng.	77	3	3	31	1 Alb.	1 Engh.	2	
	Bla.	Alb.	AnEng.	79	6‡	0	31	3 Alb. 1 Engh.		4	
	Bla.	White	Alb.	96	4	4	33	1 White		1	
	Bla.	Alb.	Alb.	101	7	0	34		2 Alb.	2	
	Engh.	Alb.	Alb.	101	5	0	No pregnancy				
	Alb.	Alb.	Engh.	120	4	0	No pregnancy				
	Bla.	Alb.	Alb.	120	2	2	No pregnancy				
	Alb.	Alb.	AnEng.	120	3	3	No pregnancy				
	Alb.	Chin.	Chin.	144	0	6	No pregnancy				
15	Chin.	Chin.	Chin.	72	5	4	31	1 Chin.		1	
	Alb.	Red	AnEng.	96	3	4	No pregnancy				

Alb., albino; Engh., English; AnEng., English Angora; Chin., chinchilla; Bla., black; Red, red; White, white.
* Ova arrested cleavage.
‡ Two of these ova arrested cleavage.

4. Other Related Observations

Although this study was mainly concerned with the effects of low temperature on fertilized rabbit ova, it revealed other biological facts which seem worth recording in this paper.

(a) *Number of Ovulations Following Gonadotrophin Injections, Number of Ova Recovered, and the Fertilization of Superovulated Ova.*—Sheep pituitary

extract was injected subcutaneously into rabbits 6 times at intervals of 12 hours in order to stimulate the growth of a large number of follicles. The extract in saline was injected in doses of 2.4 mg. (9.6 r.u.) each time. At the seventh injection, 3.2 mg. (12.8 r.u.) of the same extract was injected intravenously just after mating or insemination. Animals were killed 24 to 24½

TABLE IV

Probability of Normal Development Following the Transplantation of Ova Stored at Low Temperatures for Various Lengths of Time and Cultured at 38°C. for 24 Hours

Temperature employed	Time of storage	No. of normally cleaved ova transplanted	No. of normal young produced	Per cent of development
°C.	hrs.			
0	78	9	2	
	78	9	1	
	96	6	2	
	102	5	2	
	Total............	29	7	24
5	48	7	0	
	48	6	0	
	48	10	5	
	48	10	7	
	80	8	0	
	80	10	2	
	80	11	0	
	80	7	3	
	Total............	69	17	25
10	77	6	2	
	79	6	4	
	96	8	1	
	101	7	2	
	101	5	0	
	Total............	32	9	28
15	72	9	1	
	96	7	0	
	Total............	16	1	6.2

hours later and the ova were flushed out within the next 30 minutes. Table V presents the data obtained in these experiments.

It is quite clear that the average number of ovulations was increased about three times over normal by gonadotrophic stimulation. About 19 per cent of does produced the normal number of ova (two to thirteen) even after stimulation with gonadotrophin. However, about 81 per cent of does had super-ovulated (fourteen to sixty ova), and 36 per cent of does gave a great number of ova (thirty to sixty) following gonadotrophic stimulation.

About 15 per cent of the ova could not be recovered. This could not be ascribed only to faults of flushing or manipulation. It may have been due to (1) the loss of ova in the body cavity owing to the inefficiency of the infundibula in catching the large number of ova, or (2) to the absence of ova in the follicles.

The number of unfertilized ova was 8 per cent; fertilized but uncleaved ova, 9 per cent; 2-blastomere ova, 78 per cent; and 3–4–blastomere ova, only

TABLE V

Superovulation of Does by Gonadotrophin Injections and the Fertilization of Superovulated Ova by Mating or Artificial Insemination

Total No. of does	Total No. of ovulation points	Total No. of ova recovered				
		Unfertilized	Fertilized but uncleaved	2 blastomeres	3-4 blastomeres	Total
85	2311	162	170	1534	96	1962
Average per doe	27.2	1.9	2.0	18.0	1.1	23.1
Range	2–60	0–34	0–36	0–46	0–30	1–51

Does ovulated (less than 14 ova)................................. 18.8 per cent
Does superovulated (more than 13 ova)......................... 81.2 per cent
Does ovulated (more than 29 ova)............................. 36.5 per cent
Ova lost ... . 15.0 per cent
Ova recovered... 85.0 per cent

Unfertilized ova... 8.3 per cent

Fertilized but uncleaved ova................................. 8.7 per cent
2–Blastomere ova.. 78.2 per cent
3-4–blastomere ova.. 5.0 per cent

Sterile does... 10.6 per cent
Fertile does... 89.4 per cent
Total No. of does in luteal phase........................ 17
Total No. of sterile does in luteal phase...................... 5
No. of sterile does in luteal phase........................... 29.4 per cent

5 per cent. In view of the observations by Gregory (1930) and Pincus (1936), these figures show that the ovulation after repeated subcutaneous and a final intravenous injection of gonadotrophin, occurred in about 10 hours, similar to the situation in normal mating. However, 8 per cent of the ova were probably shed too early to be viable for fertilization or too late to meet the viable sperms and thus did not get fertilized. About 9 per cent of the ova were probably shed 1 to 2 hours late, since they were fertilized but did not cleave. About 5 per cent of the ova were apparently shed 1 to 2 hours earlier, since they had cleaved twice (Gregory, 1930).

TABLE VI

Absence of Effect of Homologous Serum and Heterologous Serum on the Viability of Fertilized Ova

Temperature	Time of storage	Homologous serum			Heterologous serum		
		Ova			Ova		
		Normal	Abnormal	Dead	Normal	Abnormal	Dead
°C.	hrs.	per cent	per cent	per cent	per cent	per cent	per cent
0	24	71	29	0	43	14	43
					50	0	50
					100	0	0
	72–80	50	50	0	69	8	23
		14	30	57	75	25	0
		33	0	67			
	96–102	40	33	26	0	41	59
					0	16	84
		36	57	7			
Mean..........		41	33	26	48	15	37
5	24	100	0	0	93	7	0
	80	59	18	24	57	21	21
		65	0	36	64	18	18
	120	0	0	100	0	44	56
Mean..........		56	5	40	54	23	24
10	24	86	14	0	80	20	0
		88	12	0	80	20	0
	72–80	65	15	20	67	33	0
	96–101	53	40	7	50	17	33
	120	57	43	0	45	10	45
		44	56	0			
Mean.........		66	30	5	64	20	16
15	48	33	29	39	80	20	0
					38	50	13
	96	0	18	82	0	33	67
Mean....... .		16	24	60	39	34	27
Mean of total...		49.6	24.6	25.8	52.1	20.3	27.0

It is of interest to note that the incidence of sterile does (does which had no ovum fertilized) following superovulation was 10.6 per cent, whereas the percentage of sterile does following normal mating has been reported as 20.4 (Hammond, 1934) and 14.2 (Chang, 1946). In view of the fact that not one of the superovulated does failed to ovulate after intravenous injection, it is most probable that the high percentage of sterile does occurring after normal

TABLE VII

The Relative Viability of Ova Fertilized by Spermatozoa of Different Males

Only ova stored at 10°C. for various lengths of time were presented.

Time of storage	No. 5 Ova			No. 8 Ova			No. 9 Ova			No. 10 Ova			No. 11 Ova		
	Normal	Abnormal	Died	Normal	Abnormal	Died	Normal	Abnormal	Died	Normal	Abnormal	Died	Normal	Abnormal	Died
hrs.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
24	67	33	0	80	20	0	83	17	0	86	14	0	86	14	0
	57	43	0	80	20	0	50	33	17	88	13	0	100	0	0
													86	14	0
72–78	65	15	20				58	17	25						
							46	38	15						
							67	33	0						
96				50	17	33							53	40	7
120							40	60	0	57	43	0	44	55	0
										45	9	45	0	20	80
144	38	19	43							0	74	26			
	35	55	10												
168	0	40	60							0	0	100			

mating is mainly due to the failure of ovulation, although some animals probably might have ovulated too early or too late. In this regard, it appears that about 10 per cent of the rabbits ovulated too early or too late after administration of gonadotrophin.

It is also of interest to note that does could be superovulated even when they were in the luteal phase as shown by the presence of corpora lutea in the ovaries. However, the percentage of sterile does at luteal phase was higher than the average of the 85 does studied (29 per cent *vs.* 10.6 per cent), and the percentage of unfertilized ova was also higher (25.4 *vs.* 8.3). In contrast,

the number of ovulations was only slightly less in the does at the luteal phase (average 22.1 *vs.* 27.2 per rabbit).

(b) *Absence of Incompatability of Serum and Fertilized Ova.*—Since pure rabbit serum was used for the flushing, storage, and culture of fertilized rabbit ova, it is important to ascertain whether or not there exists any incompatability of serum and ova from different animals.

Homologous serum (ova flushed and stored with the female's own serum) or heterologous serum (ova flushed and stored with the serum of another animal) was used throughout the experiment as a medium for storage of the ova. After storage, in most cases, the ova were cultured in heterologous serum.

Table VI presents data which show that there was no difference in the percentage of normal, abnormal, and dead ova when flushed and stored with homologous serum or with heterologous serum. In other words, there was no incompatability between serum and ova. Furthermore, there was no ill effect of heterologous serum on the subsequent development of stored ova in a normal animal.

(c) *Viability of Ova in Relation to the Spermatozoa of Different Male Animals.*— Since the spermatozoa of only 5 bucks were used to fertilize the ova of 85 does, it was thought that there might be a difference in the viability of ova owing to the fact that the ova were fertilized by the spermatozoa of different bucks. In other words, the viability of ova may be affected by the spermatozoa of a particular buck. Table VII presents the comparable data on the viability of ova fertilized by these 5 different bucks. All the ova were stored at 10°C. for various lengths of time. This table illustrates quite clearly that there was no difference in the viability of ova fertilized by the spermatozoa of different bucks. It can be postulated, therefore, that once the ovum is fertilized, its viability is independent of the male characteristics. Male gametes, apparently, play no rôle in the viability of ova.

DISCUSSION

Since the time that Milovanov (1934) found that sudden cooling had a deleterious effect which he termed "temperature shock" on spermatozoa, several investigators have confirmed this observation in different species, and have found that slow cooling prevents temperature shock (*cf.* Anderson, 1945). It was found in recent years that "temperature shock" can be prevented by diluting sperms with yolk buffer (Phillips and Lardy, 1940; Meyer and Lasley, 1945). It was rather surprising in the present experiments that ova which contain yolk were also subject to temperature shock. It seems that this observation broadens the physiological significance of temperature shock.

It is hard to say whether the damage done to the ovum in the course of

rapid cooling is in the cytoplasm or in the nucleus, or in a certain stage of mitosis. Histological examination revealed little or no difference between those ova cooled rapidly and those cooled slowly.

It was noticed during this study, however, that after various lengths of storage at low temperature, especially rapid cooling to 0°C., the cells of some ova before culture were not in good shape, and exhibited swelling, darkness, or roughness of cell membrane. But there was no great deterioration of the zona pellucida even after longer periods of storage at low temperature (Figs. 2 and 3).

It was noticed in most cases that ova with swollen blastomeres would recover normal shape or fairly good shape after 3 to 4 hours of culture at 38°C., and in some cases these ova did cleave normally. It seems that the swelling or darkness of cells was only one of the effects due to rapid cooling. There may be other mechanisms of deterioration by rapid cooling which the available histological facts could not reveal.

The harmful effect of temperature shock on the living cells has been demonstrated in the case of spermatozoa, and now in the case of ova. It was claimed by Fankhauser (1942) that temperature shock is necessary in order to let cold reach the maturation spindle before anaphase in order to produce triploidy in the newt. That sudden changes in weather may induce polyploidy in plants was shown by Belling (1925). It seems, therefore, that temperature shock must disturb normal nuclear behavior, arresting spindle formation as in the case of polyploidy or upsetting one part of the intrinsic chain reaction in nucleus or in cytoplasm at a particular stage.

Since all the facts indicate that a temperature below 15°C. was suitable for the survival of germ cells *in vitro*, and, as pointed out by Crozier (1926), a critical temperature for major biological processes is in the vicinity of 15°C., it seems quite clear that the main principal means of preservation of germ cells at low temperature is to stop or to slow down all or some of the major biological processes with resulting prolongation of the life span of germ cells.

It is interesting to note that in the ova stored at temperatures below 15°C., no cleavage occurred but there was a longer survival. At a temperature of 22–24°C., some of the ova cleaved once but all died very early. At 38°C., they cleaved normally for a longer time; *e.g.*, blastocytes were obtained in this laboratory after 5 to 6 days' culture of two blastomere ova in pure serum. The differential effect of various temperatures on the physiological reactions of living tissue is clearly shown.

The effects of low temperature on germ cells have several interesting features. When low temperature at 4–6°C. was applied to unfertilized ova of the rabbit *in vitro* or *in vivo*, parthenogenetic activity ensued (Pincus, 1939 *a*; Pincus and Shapiro, 1940). When ice was applied to the scrotal testis and epididymis

reducing the temperature to 1–3°C., the mature spermatozoa in the epididymis were disintegrated, causing separation of head and tail, but there was no harm done to spermatogenesis (Chang, 1943). Nevertheless, spermatozoa of rabbit recovered from the epididymis can be kept at 0°C. for 60 hours *in vitro* without loss of their fertilizing capacity (Walton, 1930). When a freezing mixture of ice and salt was applied to scrotal testes at a temperature of −0.8° to −2.5°C., the seminiferous tubules atrophied but no harm was done to the interstitial tissue (Chang, 1946). It is very interesting to note that the germ cells react to low temperature in such different ways depending on their physiological state and their relation to internal environment.

The effects of low temperature on spermatozoa and fertilized ova may be different. Namely, spermatozoa are haploid and their nucleus is in the resting and stable stage, while fertilized ova are diploid and their nucleus is in the active stage. In recent years, it has been found that low temperature has the same effect as colchicine in arresting spindle formation in mitotic division, thereby inducing polyploidy. That colchicine may produce monstrosities in rabbits was reported by Chang (1944).

In the application of artificial insemination in the past 20 years, no abnormality or polyploidy of livestock following low temperature treatment of spermatozoa has been reported. In the present study there was only one abnormal young developed by transplantation of 6 abnormally cleaved ova after a long period of storage at low temperature. It may have been due to the disturbance of nuclear activity by low temperature. Concerning the nuclear changes of ova after the treatment with low temperature in the present study (Figs. 1 to 19), no obvious difference has yet been noticed in the nuclei of normally cleaved ova after culture with or without previous low temperature treatment. The nuclei of most blastomeres were in the resting stage (Figs. 7 and 9); occasionally active mitosis (Figs. 8 and 10) was found in some of the blastomeres in different nuclear phases. However, many multinucleated blastomeres and distorted nuclear material were found in the blastomeres of most abnormal and retarded cleavages and in fragmented ova after culture following low temperature treatment (Figs. 11 to 14). It seems, therefore, that abnormal cleavage was due to disturbances of nuclear function. Nevertheless, once the ova withstood the low temperature without nuclear disturbance they apparently cleaved normally in culture, and developed into normal young eventually. It should also be noticed here that only 24 to 28 per cent of normally cleaved ova could develop into normal young; that is, some intrinsic disturbance was produced in apparently normally cleaved ova.

Histological sections of fertilized ova after storage at low temperatures without culture revealed that low temperature exerted its effect at all stages of mitosis (Figs. 15 to 19). Thus, all the different phases of mitosis were observed in the blastomeres although most of them were in the resting phase.

Since all the ova transplanted have been cultured at 38°C. for 20 to 24 hours, the culture for 24 hours may hinder the normal development of ova. Experiments have been undertaken to test the efficiency of transplantation, the effect of culture for various lengths of time, and the effect of low temperature storage for various lengths of time on the normal development of young. The results will be published in a subsequent paper.

Since the discovery of gonadotrophin which stimulates the growth of large numbers of follicles and, consequently, induces superovulation, many authors have investigated the probability of obtaining multiple birth by superovulation in the laboratory animals as well as in the domesticated animals. However, up to now, the results have not been promising (*cf*. Chang, 1947). The present investigation may open a new field for the more efficient utilization of the germ cells of valuable animals. Moreover, the study of genetics in mammals by experimentation, for example the physical and chemical treatment of mammalian zygotes, is handicapped by the difficulties in manipulation and the fragility of zygotes *in vitro*. Since the present investigation demonstrates that the fertilized rabbit ova can be kept *in vitro* for several days without loss of their capacity for normal development, it may offer a new approach for experimentation in mammalian genetics.

SUMMARY

Fertilized rabbit ova at the 2-blastomere stage kept in rabbit serum were stored at low temperatures for various lengths of time. They were then cultured at 38°C. for about 24 hours to determine their viability. A number of the viable ova were finally transplanted into recipient does.

It was found that rapid cooling of ova to 5° or to 0°C. was more harmful to the subsequent viability of ova than slow cooling. Rapid cooling was not more lethal to the ova than slow cooling, but did prevent their future normal cleavage. There was no difference between those ova cooled rapidly or slowly to 10°C. It was concluded that temperature shock has an adverse effect on ova, especially at the lower temperatures, though temperature shock can be remedied by acclimatization (slow cooling). Thus, the physiological significance of temperature shock would seem to be broadened.

The optimal temperature for the storage of ova was investigated. It was found that 10°C. was the best temperature; at this temperature viable ova were obtained after storage for 144 to 168 hours. At 0°, 5°, or 15°C. the ova were viable for 96 to 120 hours, while at 22–24°C., only for 24 to 48 hours.

The percentage of dead ova was low at a favorable temperature, increasing only at the end of the storage period. At an unfavorable temperature, however, the rate of death increased steadily from beginning to end of storage.

The percentage of abnormally cleaved ova (arrested cleavage and fragmentation) remained at a low level at first at a favorable temperature, but then increased just before or during death of the ova.

A critical time for the viability, the abnormal cleavage, and the death of ova was characteristic of each temperature.

About 24 to 28 per cent of the viable ova remaining after being stored at 0–15°C. for 2 to 4 days and cultured at 38°C. for 24 hours were capable of development into normal young.

The compatibility of serum and ova, the absence of a correlation between the viability of the ova and the source of the fertilizing spermatozoa, and the fertilization of superovulated ova (*i.e.*, the percentage of fertile does in follicular phase and in luteal phase, the percentage of unfertilized ova and of fertilized ova at different stages, the percentage of does that had produced a normal number of ova or had produced a large number of ova, etc.), are reported.

The possibility of a more efficient utilization of the germ cells of valuable animals by means of the present techniques, and the possibility of a new approach to the experimental investigation of mammalian genetics and development, have been mentioned.

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EXPLANATION OF PLATES

PLATE 8

FIG. 1. Photograph of fresh rabbit ova taken 24 to 25 hours after mating. (*a*) Unfertilized. (*b*) Fertilized but uncleaved. Showing two nuclei and two polar bodies. (*c*) Fertilized 2-blastomere ovum. These ova were recovered from one rabbit. × 100.

FIG. 2. Fertilized rabbit ova after storage at 0°C. for 3 days. The blastomeres of the bottom ovum are more swollen than those of the top one. × 100.

FIG. 3. Fertilized ova after storage at 10°C. for 4 days. One of the blastomeres is swollen, perhaps died. × 100.

FIG. 4. Three ova stored at 0°C. for 3 days and cultured at 38°C. for 24 hours. One dead ovum (*a*), two abnormal cleaved (*b* and *c*). × 100.

FIG. 5. Two ova stored at 5°C. for 3 days and cultured at 38°C. for 24 hours. One retarded cleavage (*a*), one normal cleavage (*b*). × 100.

FIG. 6. Three ova stored at 10°C. for 4 days and cultured at 38°C. for 24 hours. One abnormal cleavage, two of them normal cleavage. × 100.

FIG. 7. Photomicrograph of one section of an ovum stored at 5°C. for 24 hours and cultured for 24 hours at 38°C. Normal cleavage, 12-16 cell stage. Nuclei at prophase. × 325.

FIG. 8. Section of an ovum stored at 5°C. for 3 days and cultured for 24 hours. Normal cleavage, 12-16 cell stage. One of the cells in anaphase. × 325.
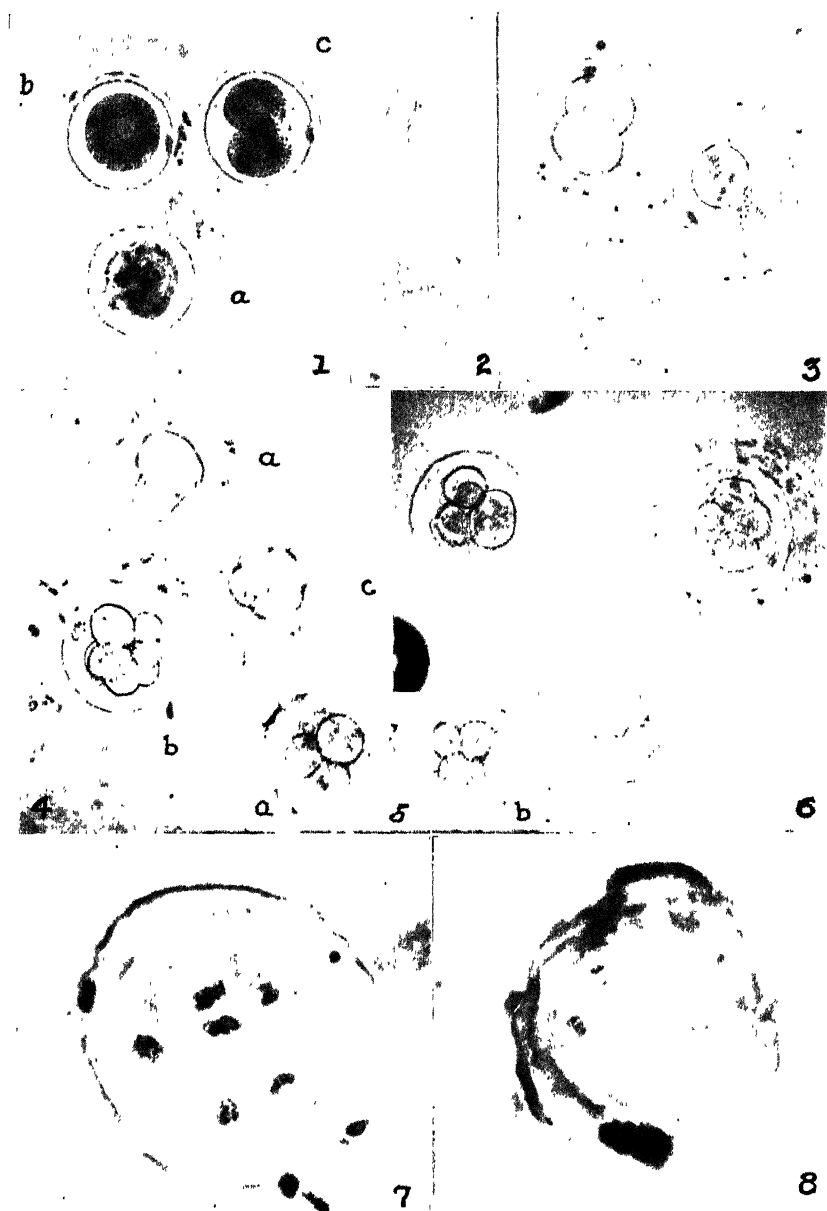
(Chang: Effects of low temperatures on rabbit ova)

PLATE 9

FIG. 9. Section of an ovum stored at 5°C. for 24 hours and cultured at 38°C. for 24 hours. 32 or more cells. Normal cleavage. × 325.

FIG. 10. Section of an ovum stored at 10°C. for 24 hours and cultured. Normal cleavage, 32 or more cells. Metaphase is shown in one of the cells. × 325.

FIG. 11. Section of two ova stored at 0°C. for 24 hours and cultured. Abnormal cleavage. Vacuoles in the cells. × 160.

FIG. 12. Section of three ova stored at 5°C. for 4 days and cultured. Retarded cleavage, 4-6 cells. × 160.

FIG. 13. Section of an ovum stored at 5°C. for 4 days and cultured. Abnormal cleavage. Showing multinuclei. × 325.

FIG. 14. Section of an ovum stored at 22-24°C. for 24 hours and cultured at 38°C. for 24 hours. Cells swollen under dissecting microscope. Showing compact cells and multinuclei. × 400.
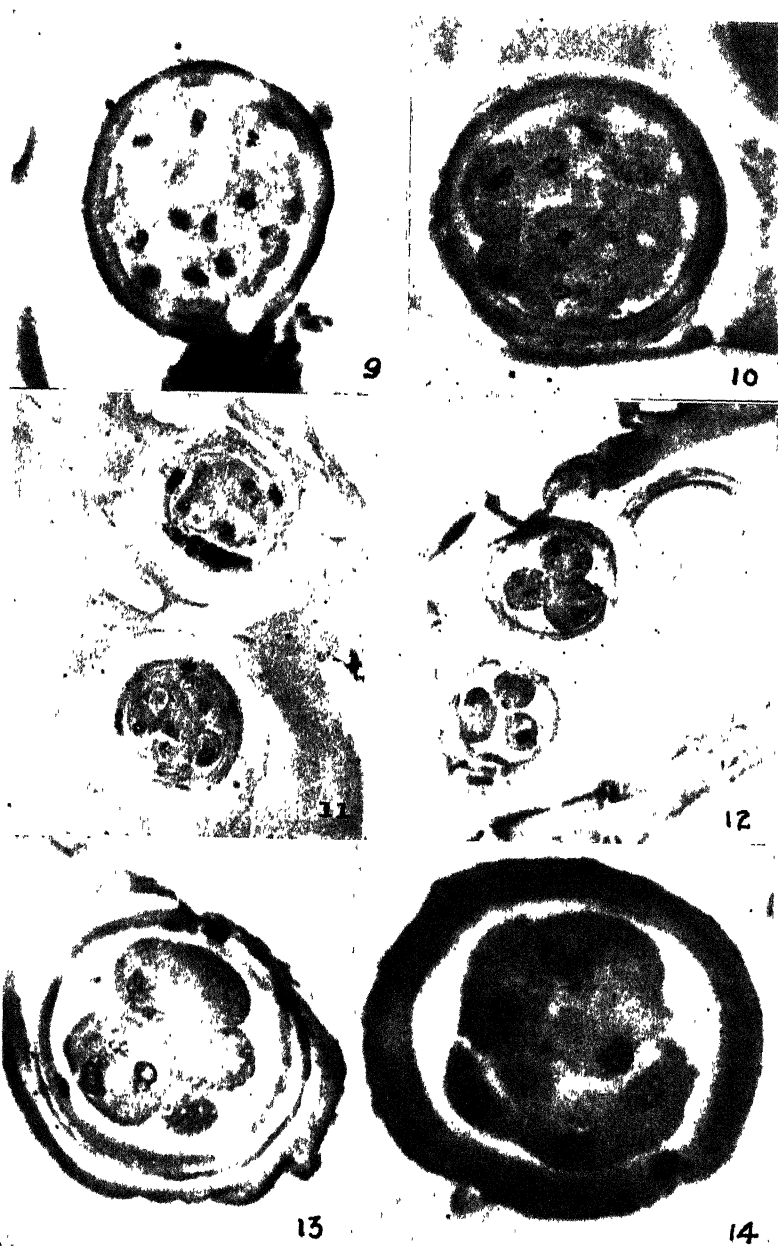
(Chang: Effects of low temperatures on rabbit ova)

PLATE 10

FIG. 15. Section of two fertilized ova stored at 0°C. for 3 days. Without further culture. × 160.

FIG. 16. Section of two fertilized fresh ova. No striking difference between Figs. 15 and 16. × 160.

FIG. 17. Section of an ovum (2 blastomeres) stored at 0°C. for 3 days, no further culture. At metaphase. × 325.

FIG. 18. Section of a fresh ovum (2 blastomeres). In the middle of anaphase. × 325.

FIG. 19. Section of a fertilized ovum (fresh) at 4 blastomere stage. Nuclei at resting. × 325.

FIG. 20. Doe with her four young. They were developed from ova stored at 10°C. for 79 hours and cultured at 38°C. for 24 hours. One English, three albino Site of incision is shown.
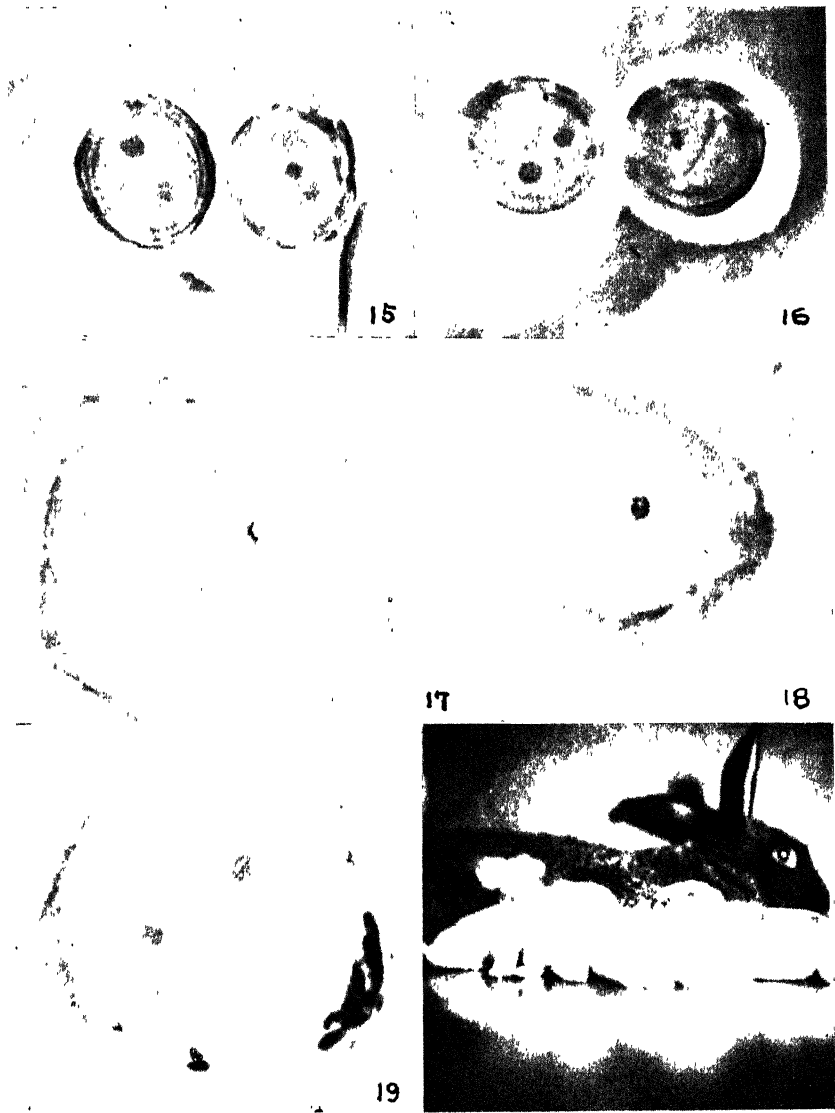
(Chang: Effects of low temperatures on rabbit ova)

THE INHIBITION OF THE ADENOSINE TRIPHOSPHATASE ACTIVITY OF ACTOMYOSIN BY MAGNESIUM IONS

By IRVIN BRAVERMAN and SERGIUS MORGULIS

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In 1939 Engelhardt and Ljubimova (2) suggested that myosin (the contractile substance of muscle) and adenosine triphosphatase are probably identical, and that the calcium ion is a specific activator of this enzyme.

Working with a myosin suspension made from rabbit muscle (by the method of Greenstein and Edsall (4) or of Weber and Meyer (11)) D. M. Needham (9) tested the activating powers of both magnesium and calcium ions and found that both activate the enzyme at pH 7.4 or pH 9.0 but that the calcium ion is much more effective than the magnesium ion.

Bailey (1), continuing and extending Needham's work, also found that the calcium ion activates adenosine *tri*phosphatase more than the magnesium ion does, but the magnesium ion activates adenosine *di*phosphatase more strongly than the calcium ion. Bailey further found that the enzymatic activity of myosin preparations is greater in a glycine buffer than in a bicarbonate-carbonate buffer. However, in either buffer the optimum activity is at pH 9.0.

The activation of adenosine triphosphate breakdown by magnesium decreases with the progressive purification of myosin. In other words, as the myosin becomes purer it loses its adenosine *di*phosphatase activity, and it is this latter activity which the magnesium ion affects most. Bailey finds that even in preparations freed from adenosine *di*phosphatase the magnesium ion still activates adenosine *tri*phosphate breakdown. However, Lohman (6) found that *only* adenosine *tri*phosphatase was activated by magnesium ions, while Ljubimova and Pevsner (5) reported that, working with myosin which had been freed from adenosine *di*phosphatase, magnesium ions actually *inhibit* adenosine *tri*phosphatase activity.

Banga and Szent-Györgyi (10) conducted their investigation on the influence of salts on the phosphatase activity with definite advantages over their predecessors. In the first place, pure myosin crystallized by Szent-Györgyi (10) was available to them; in the second place, Straub (10) had already discovered actin, which forms a complex with myosin and which was probably present as a contaminant in the myosin preparations of the previous investigators.

Banga and Szent-Györgyi found that the magnesium ion *inhibits* the adenosine triphosphatase activity of myosin but, except in very high concentrations (0.1 M), *activates* this activity of the actomyosin (a combination of actin and myosin). Still, in the presence of 0.01 M Ca ion, the Mg ion (0.001 M to 0.100 M) inhibits the action of both myosin and actomyosin. The action of Mg ions on actomyosin depends upon the concentration of K ions in the medium. Mg enhances the enzymatic activity in the presence of 0.01 M K$^+$ but inhibits it in the presence of 0.1 M K$^+$. Banga and

Szent-Györgyi explain this difference in response to Mg ions by the dissociation of actomyosin into actin and myosin in a high K ion concentration, the adenosine *tri*phosphatase activity of the freed myosin being inhibited by Mg ions.

Szent-Györgyi (10) in his comprehensive work, Studies on muscle, assumes that in the relaxing muscle, due to a high intermolecular concentration of K ions, the actomyosin undergoes dissociation and that the enzymatic activity of the free myosin becomes inhibited by Mg ions. However, in the contracting muscle, when the intermolecular concentration of K ions is decreased, the actomyosin is resynthesized and the inhibition by Mg ions is abolished. Unfortunately, Szent-Györgyi neglects his own observation *that in the presence of Ca ions the enzymatic activity is inhibited by Mg ions no matter whether the myosin is free or combined,*—a rather serious oversight!

It is also a matter of regret that Szent-Györgyi (10) gives no information as to the exact methods that he used in determining the influence of different ions on the phosphatase activity of myosin and actomyosin and does not state what type of buffer was used or what pH was maintained.

Since our experiments were performed the paper by Mommaerts and Seraidarian (7) appeared on the effects of Ca and Mg ions on the adenosine *tri*phosphatase activity of both myosin and actomyosin. The actomyosin was in solution containing 0.12 M KCl and 0.05 M NaCl and was buffered to pH 7.0 with a glycine buffer. By varying both the Ca and Mg ion concentration, they found an almost constant and maximum degree of inhibition when the molar Mg/Ca ratio exceeded 1, or even below this value. They report an inhibition of over 90 per cent even when the Mg/Ca is 0.2. Further increases in Mg ion concentration do not affect the results noticeably.

In view of these differences it seems worthwhile to report the results of our study on the effects of various concentrations of Mg ions on the breakdown of adenosine triphosphate by actomyosin in the presence of a constant concentration of K, Na, and Ca ions.

Experimental Procedure

An actomyosin sol (myosin B) was prepared from rabbit skeletal muscles according to the procedure of Banga and Szent-Györgyi (10).This sol contained 3.21 mg. per cent N and 0.240 mg. per cent P.

The Ba salt of adenosine *tri*phosphate was prepared by the method of D. M. Needham (9), and changed to the Na salt which was used in these experiments. The Na-adenosine *tri*phosphate contained 0.58 mg. per cent hydrolyzable P.

The glycine buffer of Sørenson was used which at 37°C. had a pH of 8.32.

Each experiment was set up as follows:

4.0 cc. glycine buffer

0.2 cc. actomyosin solution in 0.6 M KCl. This corresponds to 3.913 mg. actomyosin (0.642 mg. N and 0.048 mg. P). Experiments were made with variable amounts but 0.2 cc. was sufficient to yield 80 per cent decomposition in 60 minutes.

1.0 cc. KCl solution (27 mg. per cent K) giving a final concentration of 0.128 M K

1.0 cc. CaCl$_2$ solution (2.2 mg. per cent Ca) giving a final concentration of 0.002 M Ca.

1.0 cc. Na-ATP solution.
———— Variable amounts of MgCl$_2$ solution.
———— Redistilled water to make up volume to 10.0 cc.

The Ca and K concentrations in these mixtures were comparable to their concentration in rabbit muscle (8) and these were maintained constant in all experiments.

The tubes were incubated at 37°C. for 60 minutes, when the reaction was stopped by the addition of trichloracetic acid. The P was determined on the filtrate by the Fiske-SubbaRow (3) procedure using a photoelectric colorimeter.
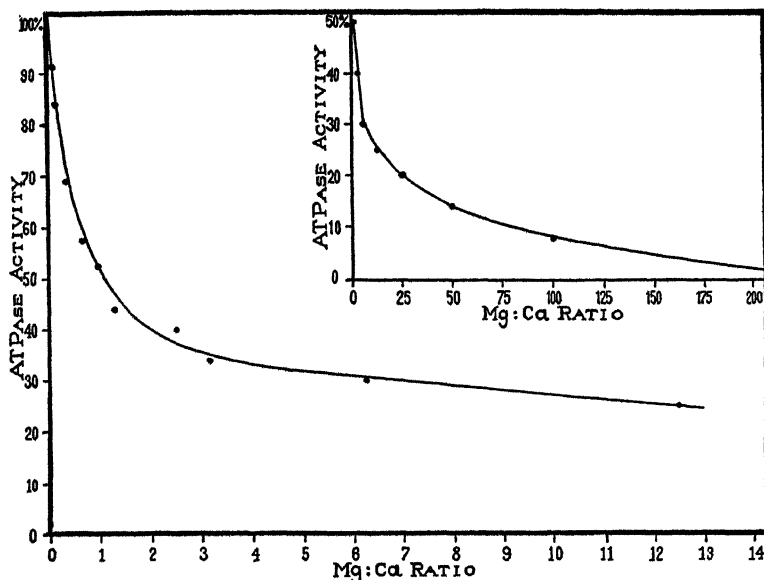
FIG. 1. Shows the relationship between adenosine triphosphatase activity and the Mg/Ca ratio.

RESULTS AND DISCUSSION

In Fig. 1 the percentages are plotted of P liberated from adenosine triphosphate by actomyosin in systems in which the only variable factor is the concentration of Mg ions. The P liberated in experiments without Mg is taken as 100 per cent. It may be noted in passing that in determining the total amount of P liberated proper corrections were made for the P set free in blank tests, but under exactly the same experimental conditions, with adenosine triphosphate without actomyosin or with actomyosin but without adenosine triphosphate (0.030 + 0.025 = 0.055 mg. P). A glance at the graph shows that the hydrolysis of adenosine triphosphate by actomyosin is inhibited over a very wide range of Mg ion concentrations. Even a trace of Mg (0.00018 M), which corresponds to an Mg/Ca ratio of 0.09, gives a measurable inhibition, while

as large a concentration as 0.4 M (Mg/Ca = 200) does not invariably cause complete inhibition. The curve, which drops very rapidly at first, approaches the abscissa asymptotically. By the time the Mg/Ca ratio has changed from 0 to 10 the adenosine triphosphatase activity is depressed about 75 per cent but further changes in the ratio from 10 to 200 occasion only a very slow depression approaching completeness.

Bearing in mind that the Mg concentration in normal rabbit muscles (8) is 0.012 M and the Mg/Ca ratio is approximately 6, we can see from the graph that the enzymatic activity of the actomyosin will be about 70 per cent inhibited. These results are in marked contrast to those of Mommaerts and Seraidarian (7) who find that the inhibition is complete long before this ratio is reached. Furthermore, according to our results, even comparatively large changes in this ratio (Mg/Ca = 6) will not very materially affect the enzymatic activity since this will vary within less than 10 per cent while the ratio may be either decreased to one-half or doubled. Contrary to the deduction which Mommaerts and Seraidarian make from their studies, namely that since the adenosine triphosphatase must be completely inhibited under the conditions existing in muscle it can have no relation to the contraction of actomyosin, our results suggest that the enzymatic activity in the normal muscle, though not optimal, is at least fairly independent of sharp alterations due to shifts in the ionic pattern. Our results also seem to indicate that the interplay of Mg and Ca ions in muscle is such as to secure greater stability of enzyme activity though not maximal activity. From the point of view of sustained muscle activity this is certainly a more advantageous arrangement.

As a corollary, it does not seem probable that adenosine triphosphatase is either absolutely inhibited or released from this inhibition through changes in Mg concentration, since any changes in intermolecular water will affect simultaneously both the Mg and Ca, and we have seen that the degree of inhibition is a function of the Mg/Ca ratio. We can, therefore, conclude that it is highly dubious whether the Mg ion could be endowed with the key function of releasing energy from adenosine triphosphate during contraction following stimulation or with conserving energy during rest.

Mommaerts and Seraidarian (7) have noted that the adenosinetriphosphatase activity of actomyosin preparations is insufficient to account for more than a very small portion of P liberated during muscle contraction. We not only corroborate this observation but we have some further evidence to support this view. Mommaerts and Seraidarian based their deduction on calculations of the amount of P set free in contractions as reported in the literature. We have unpublished data obtained in our laboratory on the P liberated from adenosine triphosphate by rabbit muscle homogenates which make it obvious that the amount P/mg. myosin/minute set free in these experiments is about a hundredfold that found in experiments with a pure actomyosin preparation.

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SURFACE INACTIVATION OF BACTERIAL VIRUSES AND OF PROTEINS*

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It has been noticed previously that certain viruses can be rapidly inactivated by shaking or by bubbling gases through the virus suspensions. Campbell-Renton (1) studied the effect of violent mechanical shaking on bacteriophages and found them to be fairly rapidly inactivated, at rates which were characteristic for each phage. Grubb, Miesse, and Puetzer (2), while studying the effect of various vapors on influenza A virus, noted that bubbling air at the rate of 1 liter a minute through the virus suspension resulted in detectable reduction in infectivity in 10 minutes. In a somewhat more extensive study McLimans (3) found that both Eastern and Western strains of equine encephalitis virus were rapidly inactivated by shaking in buffered saline suspensions. The inactivation also occurred when gases were bubbled through suspensions of the virus. The rate of inactivation was the same whether oxygen or helium was the gas used, indicating that the inactivation was probably a physical process, rather than the result of chemical interaction between virus and gas. He also noted that the rate of inactivation increased markedly as the pH was reduced from 7 to 5, though control suspensions at rest suffered no inactivation.

The inactivation of certain physiologically active proteins such as enzymes (4) and toxins (5) on shaking is a familiar phenomenon. Perhaps not quite so well known is the fact that this kind of inactivation can be specifically prevented by the presence in the diluent of very small amounts of proteins. It has been demonstrated that the spreading of a protein at a gas-liquid interface results in the denaturation of the protein, since the spread protein becomes completely insoluble in water (6) Presumably the rôle of the shaking or bubbling in the inactivation of viruses and physiologically active proteins is simply that of enormously increasing the area of the gas-liquid interface, and hence increasing the chances of the susceptible protein being spread on that surface. This paper is devoted to the kinetics of the inactivation of bacteriophage by shaking and to the effect of environmental influences on the rate of inactivation.

Materials and Methods

The group of seven *coli*-dysentery phages studied by Demerec and Fano (7) was used. The properties of this group of bacterial viruses have been summarized by

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Delbrück (8). These phages were grown on *Escherichia coli*, strain B, in a chemically defined medium of the following composition:

NH_4Cl............	1.0 gm.
$MgSO_4$.....	0.1 gm.
KH_2PO_4........................	1.5 gm.
Na_2HPO_4.......................	3.5 gm.
Lactic acid......................	9.0 gm.
NaOH.........................	about 4.0 gm. or to a final pH of about 6.5
H_2O............................	1,000 ml.

Since T_5 is not produced in the absence of calcium ion, calcium choride to a concentration of 0.001 M was added when preparing stocks of this phage. All phage stocks used contained about 10^{10} plaque forming particles per ml. All phage assays were made on strain B of *E. coli* using the agar layer technique of Gratia as modified by Hershey (9).

The saline buffer diluent used in the inactivation experiments contained 0.15 M NaCl, 0.001 M $MgSO_4$, 0.01 M buffer, and other additions as noted. Most experiments were performed using phosphate buffer at pH 6.5. Inorganic chemicals were reagent grade; the gelatin was Eastman ash-free calfskin gelatin; the bovine serum albumin was Armour's fraction V; yeast nucleic acid was a purified specimen from Eimer and Amend; the thymus nucleic acid was a highly viscous Hammarsten type preparation.

In the bubbling experiments nitrogen was passed through a coarse grade Corning sintered glass filter at the rate of 1 liter per minute producing a vigorous effervescence in the virus suspension held in the filter. The gas was saturated with water vapor and the gas stream as well as the filter and its contents was in a constant temperature bath.

The shaking experiments were carried out in test tubes 15 mm. × 100 mm. with a capacity of 16 cc. These tubes as well as dilution tubes were cleaned with hot acid dichromate, well rinsed, and twice boiled with distilled water. Pipettes were similarly acid-cleaned and rinsed with hot distilled water The test tubes were closed with rubber stoppers which were boiled with sodium hydroxide, well rinsed, then twice boiled with distilled water before each use. The most meticulous cleanliness was essential in obtaining consistent results. The shaking machine had a horizontal reciprocating motion of 320 cycles per minute and the carriage traversed a distance of 7 cm. The test tubes were shaken with the long axis parallel with the direction of motion of the carriage.

EXPERIMENTAL

Kinetics of the Inactivation Reaction.—Bacteriophage T_7 at an initial concentration of 6×10^9 plaque-forming particles per ml. was diluted in the saline-buffer diluent to a concentration of about 10^4/cc. The conditions of the experiment were: phosphate buffer of pH 6.5, temperature 26°C., volume of phage suspension 5 cc., shaker stopped every 2 minutes for sampling. The log per cent survivors proved to be a linear function of time indicating that the rate of

phage destruction was proportional to the concentration of surviving phage;

$$-\frac{dP}{dt} = KP$$

or $K = 1/t \ln P_0/P_t$

The data of this sample experiment are given in Table I. The first order velocity constant for the inactivation of T_7 under the stated conditions was 0.28 min.$^{-1}$. There was no recovery of activity on standing in buffer diluent or broth, and inactivation occurred at a significant rate only during the periods of shaking (Table I).

TABLE I

The Inactivation of T_7 Bacteriophage by Shaking in Saline-Buffer Diluent at 26°C. and pH 6.5

Time	Sample	Count	Survivors per 0.1 ml.	P_0/P_t	$\ln P_0/P_t$	K
min.	*ml.*					
0	0.02	136				
			Av. 753			
0	0.02	165				
2	0.05	225	450	1.67	0.51	0.26
4	0.05	104	208	3.6	1.28	0.32
6	0.05	68	136	5.5	1.7	0.28
8	0.05	45	90	8.3	2.1	0.27
10	0.1	52	52	14.4	2.7	0.27
15	0.1	13	13	58	4.1	0.27
35	0.1	1	1	753	6.6	(0.19)
						Av. 0.28

The velocity constants for the inactivation of each of the seven *coli* phages and of two of their mutants at pH 6.5 and 26°C. are given in Table II.

From the data in Table II it may be noted that the small phages T_1, T_3, and T_7 are inactivated more rapidly than the larger phages. Phage T_{4r+} and its rapid lysing mutant (10) T_{4r} are much more stable than the other phages. With both T_2 and T_4 phages there was no significant difference between the stabilities of wild type and rapid lysing mutant. Also in mixtures of wild type and mutant forms, the proportion of the two types remained constant during the inactivation.

The volume of phage suspension was varied from 4 cc. to 7 cc. per 16 cc. tube without affecting the velocity of the inactivation. However, if the tube is filled with virus suspension so that no air space is left, there is no perceptible inactivation of the phage during 40 minutes' shaking, even when half a dozen glass beads are added to the tube. Because of the possibility of inactivation

of phage through adsorption to the glass walls of the tube or to the rubber stopper, both of these surfaces were coated with melted paraffin. In the paraffin-coated tube the rate of inactivation of phage was the same as in uncoated tubes even though the paraffin surfaces were not wetted by the suspension of phage. If loss of activity were due to adsorption, the virus must adsorb equally well to glass and to paraffin. From these experiments it would appear that the shaking or agitation of the fluid suspension in contact with glass surfaces is not the cause of the inactivation of virus, but rather that the inactivation occurs at the gas-liquid interface which is present in enormous area when tubes half filled with liquid are violently shaken.

The variation of velocity constant of inactivation as a function of pH is shown in the curves of Fig. 1. From these curves it is evident that the rate of inacti-

TABLE II

The Average Velocity Constants for the Shaking Inactivation of coli Phages at 26°C. and pH 6.5

Phage	Velocity constant
T_1	0.59 min.$^{-1}$
T_{2r+}	0.24
T_{2r}	0.23
T_3	1.2
T_{4r+}	0.05
T_{4r}	0.07
T_5	0.24
T_6	0.20
T_7	0.48

vation by shaking is minimal between pH 5 and 8 but increases rapidly outside this range.

The small phage T_7 is much more rapidly inactivated at all pH values than are the larger phages, and repeated assays at a given pH are less reproducible with the small phages, resulting in a more erratic looking curve. All phages were markedly unstable in the absence of shaking at pH 3 except T_4. At pH 4 the phages were more stable than at pH 3 but in a few cases there was a 10 to 50 per cent loss in activity on standing at room temperature for 1 hour. At the higher pH values studied there was no detectable loss in activity in unshaken controls during the course of the experiments. Phage T_7 which is very rapidly inactivated by shaking at pH values above 7 is not detectably inactivated in unshaken control tubes after 1 hour at pH 8.7.

The effect of temperature on the velocity constants of inactivation was determined for phages T_1 and T_7. The averages for a number of determinations at 0°, 25°, and 38°C. are given in Table III.

From these values, using the Arrhenius equation, the Arrhenius constant

for the shaking inactivation of phages T_1 and T_7 appears to be about 10,000 cal./mol. This value is higher than the reported values for the heat of activation of denaturation of proteins by urea and by shaking (11) and far less than the values for heat denaturation of proteins.

In the presence of 1 mg./ml. of gelatin all phages were stable on shaking for 1 hour at room temperature at pH 6.5. Therefore, the protective effect of
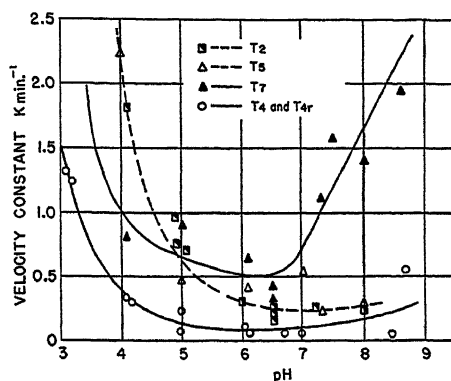
FIG. 1. Velocity constants, K minute^{-1}, as a function of pH for the phages T_2, T_4, T_5, and T_7 at about 26°C.

TABLE III

. *The Average Velocity Constants for Inactivation of T_1 and T_7 at 0°, 25°, and 38°C.*

Temperature	Velocity constants for	
	T_1	T_7
°C.		
0	0.31	0.09
25	0.59	0.48
38	0.97	0.70

various concentrations of gelatin on phage T_5 was determined. The results are summarized in Fig. 2.

It will be seen from Fig. 2 that as little as 0.01 γ per ml. of added gelatin has a definite protective effect on phage T_5 while 1 γ/ml. gave complete protection for 14 minutes. However, after 20 minutes of shaking with 1 γ/ml. of gelatin the phage activity began to decrease. It would appear that the duration of the protective effect of gelatin is a function of the concentration of gelatin, and that the gelatin also appears to be inactivated by shaking. If the survival time of the gelatin is taken as the time when the inactivation curve becomes parallel to the inactivation curve in the absence of gelatin, then it becomes possible to

estimate the rate of disappearance of the gelatin. The disappearance of the gelatin under these conditions appears to follow the kinetics of a first order reaction with a half life of about 2 minutes. This relationship does not hold for concentrations of gelatin above about 0.5 γ/ml. since as the protein concentration becomes higher the kinetics change from those of a first order reaction to those of a zero order reaction in which the rate of inactivation is determined
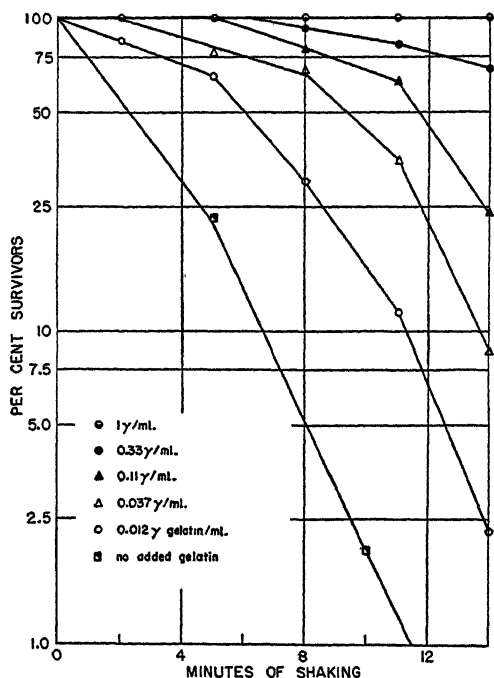
FIG. 2. The inactivation of phage T$_5$, shaken in the presence of various amounts of gelatin.

by the available surface rather than by the concentration of protein in solution (11).

If this supposition is correct, preshaking of the gelatin solutions before adding the phage should destroy the protective effects of the gelatin. The experiment illustrated in Fig. 3 is identical with the previous experiment except that the dilutions of gelatin in saline-buffer diluent were preshaken for 15 minutes before addition of phage. Then after phage addition the tubes were shaken and samples withdrawn at intervals for assay. It may be seen from Fig. 3 that the protective effects of all quantities of gelatin through 0.33 γ/ml. are destroyed by shaking for 15 minutes so that the resultant inactivation curves are identical with the curve with no added protein. There is little appreciable

diminution in the protective effect of 1 γ/ml. of gelatin after 15 minutes of shaking.

Similar experiments have been carried out using various concentrations of gelatin with T$_7$ and T$_{2r}$, with very similar results.

Since gum arabic is a colloidal substance with reputed protective effect against inactivation of tuberculin (12) it was tested for its effect on the shaking
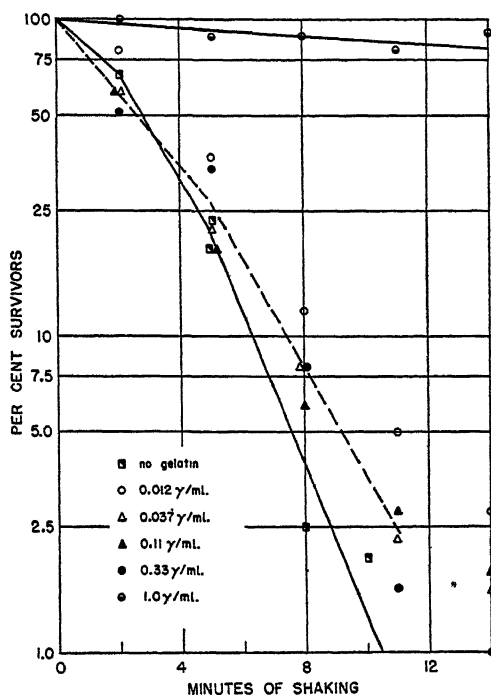
FIG. 3. The inactivation of phage T$_5$, shaken in the presence of various amounts of gelatin which had already been shaken for 15 minutes before addition of phage. The symbols correspond to the same gelatin concentrations as in Fig. 2. The solid line is the curve for no added gelatin and the dotted line is an average curve for the tubes containing added gelatin.

inactivation of phage T$_5$. As may be seen from Fig. 4 gum arabic gives a family of curves similar to those given with gelatin, except that about 100 times as much gum arabic is required to equal the effect of gelatin. It is probable that the protecting effect of the gum arabic is due to contamination with about 1 per cent of protein. This agrees with previously made quantitative estimates of the protective effect of gum arabic against the surface inactivation of tyrosinase (13).

In a similar manner yeast nucleic acid and thymus nucleic acid prepared

according to Hammarsten were tested for possible protective effect. Both of these substances had a protective effect equivalent to about 1 per cent of their weight of gelatin. Since no amino acid analyses were available for these samples of nucleic acid we cannot say whether the protective effect is due to contamination with protein or is an inherent property of nucleic acids.

A commercial sample of bovine serum albumin (Armour fraction V) prepared by alcohol fractionation was tested for its protective effect with the results shown in Fig. 5.
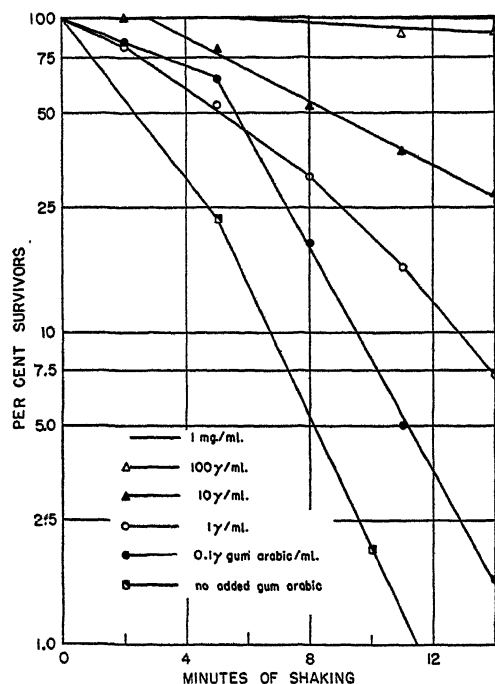
FIG. 4. The inactivation of phage T₅, shaken in the presence of various amounts of gum arabic.

By a comparison of the curves of Fig. 5 with the curves with gelatin in Fig. 2 it may be seen that serum albumin is about one-tenth as active in protecting the virus from inactivation as is gelatin. This observation is in accord with experiments of Berger, Slein, Colowick, and Cori (14) on the inactivation of hexokinase in which the protective effect of serum albumin was about one-tenth that of insulin or rabbit muscle protein. It also agrees qualitatively with reported effects on the stability of diphtheria toxin diluted for the Schick test. Edsall and Wyman (15) reported that 500 γ/ml. of human serum albumin gave incomplete protection while 1 mg./ml. gave excellent protection. Moloney and Taylor (16) using similar test conditions reported that 12.5 γ/ml. of gelatin gave

considerable protection while 25 γ/ml. of gelatin gave complete protection for 6 months.

If the inactivation of bacteriophages is due to some change occurring at the surface of gas bubbles produced in the fluid by shaking, this same kind of inactivation should occur when an inert gas is bubbled through a suspension of the virus. Accordingly 25 ml. of buffer-diluent at pH 6.5 containing phage T_{2r} at a concentration of 2.5×10^4 infectious particles per ml. were placed in a Corning sintered glass filter of coarse grade. This was held in a water bath at 30°C. and
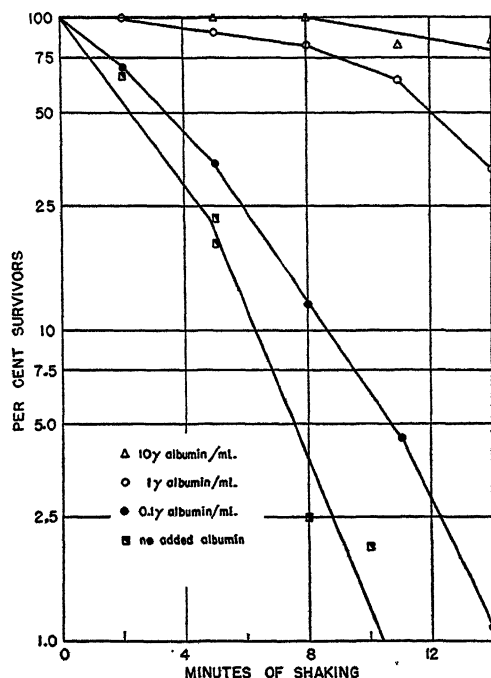
FIG. 5. The inactivation of phage T_5, shaken in the presence of various amounts of bovine serum albumin.

nitrogen gas was bubbled through the filter at the rate of 1 liter per minute. Samples were withdrawn at 5 minute intervals for an hour without interrupting the gas flow. The inactivation followed the kinetics of a first order reaction throughout this time with a velocity constant of 0.047 minute^{-1}, as compared with 0.23 minute^{-1} for shaking with air at 26°C. Similar results were obtained with T_7 although the rate was somewhat faster with this phage.

DISCUSSION

The denaturation of proteins probably involves the unfolding of a highly specific globular structure into a relatively unspecific polypeptide chain. This

change exposes hitherto hidden -SH and phenolic groups to the action of chemical reagents, and results in the loss of solubility and of the specific physiological activity of the protein. Denaturation may be brought about by the action of heat, of chemicals such as urea, of detergents, of excessive concentrations of H^+ or OH' ions, and by shaking. All of these denaturing agents will also bring about a destruction of the infectious properties of viruses.

That vigorous shaking will cause the precipitation of proteins from solution has been known for a long time. The precipitation of egg albumin from 1 per cent solutions on vigorous shaking follows the course of a zero order reaction since the high concentration of protein maintains the gas-liquid interface in a saturated condition. The rate-limiting factors are the amount of surface available, and the rate at which the surface is renewed by agitation (11) With highly diluted proteins however, one might expect the kinetics of the reaction to be first order since the number of protein molecules arriving at the surface in unit time will be proportional to the protein concentration. There is very little data on this point in the literature. Shaklee and Meltzer (4) in 1909 studied the effect of shaking on the stability of pepsin in HCl. From their data it can be calculated that the inactivation of pepsin follows the course of a first order reaction with a velocity constant of 0.029 minute^{-1} at 33°C. Since no characterization of the pepsin was made it is impossible to say how much pepsin was present or even how much total protein was present in the shaking experiments. However, it is significant that the addition of peptone stabilized the pepsin, there being a loss of only 25 per cent of the pepsin activity on shaking for 24 hours at 33°C. in the presence of peptone. Shaklee and Meltzer made certain observations that agree closely with our own observations on the inactivation of bacteriophage by shaking, namely:

1. Presence of glass beads did not accelerate shaking inactivation.
2. No inactivation of shaking full bottles, with or without glass beads.
3. Results in paraffined bottles were same as in non-paraffined glass bottles.
4. Results in sealed glass tubes were same as in rubber-stoppered bottles.
5. Inactivation rate increased with increasing acidity.
6. Results were the same with air, CO_2, or H_2 as the gas phase.

MacFarlane and Knight (17) in 1941 studied the α toxin of *Cl. welchii* which they demonstrated to be an enzyme, lecithinase. This enzyme when highly diluted was rapidly inactivated by bubbling air or nitrogen through the enzyme solution. They did not follow the course of the inactivation over a sufficient range of activities to make it possible to decide whether the kinetics are those of a zero order or a first order reaction.

It has been observed repeatedly that physiologically active proteins on high dilution often show a spontaneous loss of activity which may be prevented by carrying out the dilution procedure in the presence of other proteins. In Table IV is listed a number of examples of this phenomenon culled from the literature. Included are the concentration at which the activity of the protein in question

is measured and at which the inactivation is observed, together with the concentration of added protein which has been found to prevent this inactivation.   It

TABLE IV

A Summary of Data from the Literature Concerning Physiologically Active Proteins Which Are Unstable When Highly Diluted, Including the Concentration at Which the Protein Is Usually Assayed and at Which Its Lack of Stability Is Noted, and the Concentration of Protective Protein Employed to Stabilize It

Physiologically active protein	Concentration at which protein is markedly unstable	Concentration of protective protein employed	Reference
Diptheria toxin in Schick test	0.02 to 0.2 γ/ml.	1mg./ml. serum albumin 25 γ/ml. gelatin	15 16
Tetanus toxin	M. L. D. is 3 $\times$ $10^{-4}\gamma$ protein	10 mg./ml. peptone*	18
α toxin of *Cl. welchii* or lecithinase	M.L.D. is 0.2 to 0.5 γ protein	5 mg./ml. gelatin* 10 mg./ml. serum albumin*	19 20
Botulinus toxin	M.L.D. is 10^{-4} γ protein	2 mg./ml. gelatin*	21
Invertase	—	2 to 4 γ/ml. gelatin	22
Tyrosinase	1 γ/ml.	10 γ/ml. gelatin	13
Ascorbic acid oxidase	1 γ/ml.	6 γ/ml. gelatin	23
Carbonic anhydrase	1.6 γ/ml.	33 γ/ml. peptone	24
Catalase	<3 γ/ml.	—	25
Desoxyribonuclease	3 γ/ml.	100 γ/ml. gelatin*	26
Hexokinase	4 γ/ml.	6 γ/ml. insulin or 60 γ/ml. serum albumin	14
α glycerophosphate dehydrogenase	2.5 γ/ml.	1 mg./ml. gelatin*	27
Bacteriophage	10^4 particles/ml.	1 to 10 γ/ml. gelatin	

* Protective effect not titrated, concentration given is lowest one tested or only one given in reference cited.

should be noted that in many cases the concentration given is the lowest concentration of protein tested for protective effect since no titration of the protecting protein was made.   It may also perhaps be significant that many of the enzyme activities are assayed in a Warburg or similar manometric apparatus in which a

vigorous shaking of a highly diluted enzyme preparation is part of the assay procedure.

From Table IV it may be noted that the physiologically active proteins with which this type of instability has been observed are all proteins in which the specific activity is measured at a final protein concentration of 4 γ/ml. or less. Presumably proteins which must be assayed at higher concentrations do not show this phenomenon. Also it may be noted that where the protecting protein has been assayed, the amount required has varied from 1 γ/ml. of gelatin in the case of short duration experiments with bacteriophage to 25 γ/ml. of gelatin needed to stabilize Schick toxin for 6 months at room temperature. Serum albumin when it has been compared with other proteins such as insulin or gelatin has been much less effective as a protecting agent. It is highly significant that proteins present in solutions of less than a few γ/ml. concentration are highly unstable, and that they are protected from inactivation by the presence in solution of other proteins at a concentration higher than a few γ/ml. It has been shown that proteins will unfold at a gas-liquid interface to form a monomolecular film about 10Å thick and covering an area of about 10 cm.^{2}/γ of protein (28). This protein film is insoluble in water, and once formed on a quiet surface will effectively prevent more protein molecules of the same or different type from reaching the surface. On stirring or agitation of the surface however, the protein film will be folded upon itself to form an insoluble coagulum of denatured protein, leaving a fresh interface for the unfolding of additional protein. A physiologically active protein present at a concentration of 1 γ/ml. could then be completely spread and inactivated at a total interface corresponding to 10 cm.^{2}/ml., an area readily obtainable with very little shaking. In the presence of a second protein, the rate of inactivation of the physiologically active protein would be a function of the relative concentrations of the two proteins, of their respective diffusion constants, and of the relative ease with which they unfold once they reach the surface. A protective protein present at a concentration of 10 γ/ml. should effectively exclude from the surface a protein of similar properties present at a concentration of 1 γ/ml. Also if a physiologically active protein is present at a concentration of 10 γ/ml. or higher, the available surface will be saturated with an undetectably small fraction of this protein and hence no loss in activity will be noticed unless the shaking is more violent and prolonged than in the usual assay procedures in the Warburg apparatus for instance.

Langmuir and Schaefer (29) derived an equation for the diffusion of solute molecules to the surface, assuming only that every molecule which reached the surface stayed at the surface. This is a reasonable assumption for protein molecules if every molecule which reaches the surface unfolds into a film. The equation is

$$n = 2 \, n_0 \left(\frac{Dt}{\pi} \right)^{1/2}$$

where n is the amount of protein reaching 1 cm.[2] of surface in time t, n_0 is the concentration of protein per cm.[3] and D is the diffusion constant of the protein. For egg albumin at 20°C. and a concentration of 100 γ/ml., the surface should be saturated in 1 second, whereas at a concentration of 5 γ/ml. it would take 26 minutes to saturate the surface. Bull (30) measured the rate of fall of surface tension with time in solutions containing various concentrations of egg albumin. At albumin concentrations higher than 50 γ/ml., the major portion of the surface tension drop occurred in less than a minute, while at a concentration of 5 γ/ml. there was no noticeable drop for several minutes, then the major fall in surface tension occurred between 5 and 15 minutes, the surface tension approaching the equilibrium value in 30 minutes.

At a concentration of egg albumin of 1 γ/ml., the albumin will reach the surface in the quantity of 10^{-2} γ per cm.[2] of surface in 100 seconds. If the surface to volume ratio is increased by shaking or bubbling it is obvious that a large proportion of the total protein would reach the surface in a fairly short time especially since Langmuir and Schaefer (29) point out that in stirred solutions the amount of solute reaching the surface is proportional to time rather than to the square root of time as it is in solutions at rest.

Failure to realize that the concentration of protein in solution was the critical factor in determining whether or not rapid spontaneous inactivation occurred on dilution, has resulted in the publication of probably erroneous conclusions. For instance Traub, Hollander, and Friedemann (31) concluded that broth and serum "potentiated" the lethal action of tetanus toxin. They considered the possibility that the added broth or serum prevented the inactivation of tetanus toxin but discarded this explanation, largely on the grounds that if the low titer of toxin in saline were due to inactivation it would have to occur with unreasonable rapidity, and because "potentiation" occurred in the case of titrations in small animals such as mice but not in large animals such as rabbits. Examination of their data reveals that with toxin lot 1556 the lethal dose in rabbits is 0.1 ml. of a 1/10 dilution in either broth or saline; whereas in the guinea pig the lethal dose is 0.1 ml. of a 1/2000 dilution in saline, and 0.1 ml. of a 1/128,000 dilution in serum. It seems not unreasonable to assume that culture filtrates containing tetanus toxin when diluted beyond 1/2000 in saline contain less than the critical 1 γ/ml. of protein; especially since in the titrations recorded in their paper, the *potentiating* effect of broth decreased to almost nothing when the broth was diluted 1/1000 in saline. The observations of Traub *et al.* on potentiation can be satisfactorily explained on the assumption that tetanus toxin is markedly unstable when it is diluted beyond a limiting value for total protein concentration, and that dilution in the presence of small amounts of protein prevents this loss of activity.

In the present paper we have discussed the inactivation of viruses by shaking as a process quite analogous to the surface denaturation of proteins. We do not

picture the inactivation of the virus as necessarily involving an unfolding of the entire virus particle into a protein layer 10Å thick. In fact Seastone has shown (32) that tobacco mosaic and vaccinia viruses do not readily unfold in the way that egg albumin does, but that never the less these viruses do form surface films. We merely suggest that once the virus reaches a gas-liquid interface it is subjected to such forces that it may very rapidly be deprived of the property of infectivity. This loss of infectivity may be prevented by saturating the gas-liquid interface with another protein, thereby denying the virus acess to the surface. In this respect the phenomenon is analogous to the surface denaturation of proteins.

The prevention of surface denaturation is not the only protective rôle which may be played by proteins. Sumner (33) has demonstrated that dilute solutions of crystalline urease are rapidly inactivated by traces by heavy metals. This type of inactivation can be prevented by the addition of proteins, as well as by gum arabic, hydrogen sulfide, amino acids, and many other substances. Urease can similarly be protected by proteins against inactivation by small amounts of oxidizing agents. Proteins should play a similar rôle in the protection of viruses against the inactivating effects of heavy metals and oxidizing agents. It is probably a summation of these various protective mechanisms which is responsible for the generally recognized fact that viruses are more stable when diluted in serum or broth than when diluted in salt solutions or distilled water.

SUMMARY

1. The seven bacterial viruses of the T group active against *E. coli*, are rapidly inactivated at gas-liquid interfaces.

2. The kinetics of this inactivation whether brought about by shaking or by bubbling with nitrogen are those of a first order reaction.

3. This inactivation may be prevented by the addition of enough protein to maintain the gas-liquid interface in a saturated condition.

4. The analogy between this phenomenon and the surface denaturation of proteins is pointed out and discussed.

Addendum.—Since submitting this manuscript, we have found a paper by J. Steinhardt (34) on "The stability of crystalline pepsin" in which the inactivation of pepsin by shaking is noted. At a pH of 6, temperature of 25°C., and pepsin concentration of about 30 to 60 micrograms per ml., pepsin is inactivated by shaking in accordance with the kinetics of a first order reaction. The velocity constant was independent of pH over the range of 4 to 6 but was somewhat dependent on the rate of shaking. The inactivated pepsin separated from solution as an insoluble suspension.

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THE RELATION BETWEEN VISUAL ACUITY AND BRIGHTNESS DISCRIMINATION*

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I

INTRODUCTION

The capacity for seeing fine detail is called "visual acuity" or "resolving power of the eye," and is defined quantitatively as the reciprocal of the angle, in minutes, occupied by the detail that can just be discriminated. The test object must be specified, because different test objects yield widely different numerical values of visual acuity. The prevailing illumination has a profound effect on visual acuity. At low and moderate brightnesses, visual acuity increases with illumination in a characteristic way, approaching a maximum value which remains constant with further increase in brightness (see Shlaer, 1937, for references).

Another major factor in visual acuity is the contrast between object and background, and it is with this that the present paper is concerned. In most visual acuity tests contrast is at a maximum, the test object being black and the background white; but in everyday seeing, objects and their backgrounds are usually much closer in brightness. With reduction in contrast, visual acuity also decreases. This fact has been known for a long time (see Aubert, 1865, p. 198 ff.; Roelofs and Bierens de Haan, 1922) but quantitative and carefully controlled experimental work has been done only comparatively recently. Cobb and Moss (1928) measured visual acuity as a function of contrast, over a wide range of contrasts. They studied only a few rather high brightnesses. Conner and Ganoung (1935) extended these researches to lower brightnesses, but their data with low contrast test objects are too scanty to yield the necessary information. Byram (1944) has measured the threshold for seeing lines and circles of varying degrees of contrast, at high outdoor levels of illumination.

Studies of the effect of test field size on brightness discrimination are somewhat comparable to the visual acuity studies, though the criterion is the detection of contrast, rather than the resolution of shape. Lasareff (1911) and Heinz and Lippay (1928) worked at a single high brightness. Steinhardt (1936) covered the whole significant brightness range, but did not study very

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* This work was reported to the Optical Society of America in October, 1946 (Hendley, 1946).

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small sizes.   Holway and Hurvich (1938) and Crozier and Holway (1939) did not investigate sizes below 1°.   Graham and Bartlett (1940) extended the results to smaller sizes, covering the significant range of cone brightnesses.   Blackwell (1946) has published measurements made over a very wide range of sizes and brightnesses.   Lamar, Hecht, Shlaer, and Hendley (1947) studied the significant range of sizes and shapes, at high brightness only.

This study was undertaken to give a somewhat more comprehensive view of the relation between size, contrast, and brightness in the determination of visibility.   Visual acuity thresholds were measured as a function of background brightness and contrast.   The measurements were made by selecting a target size and background brightness, and varying the target brightness until the minimum contrast was found at which the test object could be resolved.   The threshold difference in brightness between target and background, divided by the background brightness, is called the contrast fraction, $\Delta I/I$.

When an adequate range of sizes and brightnesses was thus investigated, a body of data was obtained from which the following information was derived:

1. The relation of $\Delta I/I$ to background brightness, for ΔI fields of various sizes, the criterion of seeing being the recognition of the orientation of the target.

2. The relation between visual acuity and brightness at various contrasts between test object and background.

3. The relation between visual acuity and contrast at various levels of illumination.

4. The relation between contrast and the frequency of seeing the test object against the background.

II

Apparatus and Procedure

1. *Apparatus and Calibrations.*—In all the present measurements the observer looks monocularly through an artificial pupil at a uniformly illuminated field 30° in diameter, whose brightness may be varied over the whole visual range.   In the center he sees the configuration shown in Fig. 1.   The target is a central rectangle of light twice as long as it is wide, whose size, brightness, and orientation may be varied to secure the appropriate measurements.

The optical system for controlling the various parts of the field is shown in Fig. 2. It is a modified form of Shlaer's apparatus (Shlaer, 1937) for measuring visual acuity, and was actually modified by Dr. Shlaer himself.   The light source S is the incandescent ball of a tungsten arc.   An image of the source is focussed by the condenser C on the projection lens system $P.L.$ The light passes from $P.L.$, which serves as a secondary source, through the half-silvered mirror M_1.   (The beam reflected at M_1 will be considered later.)   The undeviated transmitted beam is reflected from a front-surface plane mirror M_2, not shown in the diagram, to the field lens $F.L._1$.   This lens focusses an image of $P.L.$, the secondary source, at a point just behind the artificial

pupil P, a circular aperture 2 mm. in diameter. Thus $F.L._1$ appears evenly illumi-
nated to the eye, and provides the background field I against which the test object is
viewed. This field subtends 4° at the subject's eye.

The test object ΔI is provided by the beam reflected at the mirror M_1, which is
focussed by L to give an image of C at the wedge W. The beam is so narrow at the
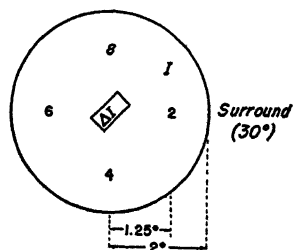
FIG. 1. A diagram of the field as the subject sees it. The surround has the same
brightness as the I field. Its edge, 15° from the center, is not shown.
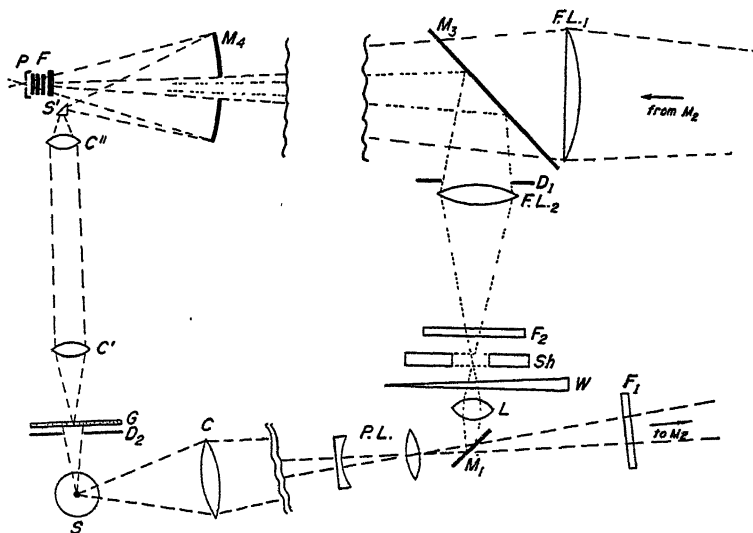
FIG. 2. Diagrammatic top view of the apparatus, not drawn to scale

wedge that no balancer is necessary. The beam then diverges, passing through the
shutter and a neutral filter, to the field lens $F.L._2$, which brings it to a focus again at
the exit pupil P, after being reflected from the half-silvered mirror M_3. The eye thus
sees an enlarged image of the source as the ΔI field, superimposed on the center of the
background field.

The size and shape of the ΔI field is controlled by a series of eight rectangular metal
diaphragms at D_1. The length of the diaphragm opening is twice its width. The

smallest rectangle is 2' wide; the others range up to 50', in steps of 0.2 log unit. The diaphragm holder can be rotated to present the ΔI field in different positions; detent stops are provided at the four positions used in the investigation.

The two field lenses are equidistant from the observer, at 1 meter. Thus when the observer focusses on the test object he is also in focus for objects on the surface of $F.L._1$. This fact is made use of in providing reference marks to aid the observer in keeping proper focus, and in judging the orientation of the test field. Small black numbers, legible only when in focus, are placed 1.25° from the center of $F.L._1$, on the vertical and horizontal axes. The four positions in which the target is presented are horizontal, vertical, diagonally to the right as in Fig. 1, and diagonally to the left.

When the test field is small, it must be surrounded by a large area of approximately the same brightness if the eye is to give its best performance. This is particularly true at high brightness (Lythgoe, 1932; Steinhardt, 1936). In this apparatus a surround of 30° is provided by the spherical front-surface mirror M_4, which has a hole in its center to admit the light from the I and ΔI fields. The source S illuminates the ground glass G, through the adjustable iris diaphragm D_2. The ground glass is imaged by the condensing lenses $C'C''$ at the right-angled prism S', whence it is reflected, diverging to fill the mirror M_4. This mirror focusses an image of S' at the pupil P, and is therefore seen as evenly illuminated. By varying the opening of the iris D_2, the surround brightness may be equated with the I field. The black circle in Fig. 1 marks the boundary between the I field and the surround.

Close to the artificial pupil are the neutral Wratten filters F that control the brightness in steps of about 0.3 log unit over an intensity range of over 8 log units. There is also space at the eyepiece for a removable filter; here the Wratten Number 70 red filter (shown as the large filter at F in Fig. 2) is inserted when red light is to be used. At F_1 and F_2 in Fig. 2 neutral filters can be inserted to control the relative brightness of the I and ΔI fields.

A fixation point is needed to orient the observer for off-axis vision at low brightness. This is provided by a small light bulb placed between the eyepiece and the mirror M_4, so that the mirror forms a virtual image of the filament in the plane of the test object. The side of the bulb nearest the observer is painted black, so the filament cannot be seen directly.

The exposure of the ΔI field is controlled by an electric clock motor, actuating a solenoid which opens and closes a photographic shutter. The target is exposed for 3 seconds out of every 10; thus there are six exposures a minute.

The wedge and all the neutral filters were calibrated with a Martens polarization photometer, with light of the same color temperature as that used in the experiments. The calibrations were then repeated with light transmitted by a No. 70 Wratten filter. The absolute brightness calibration was made by comparing the I field with one that could easily be measured directly (cf. Lamar et al., 1947). The brightness of the ΔI field was found by matching it with the I field at one wedge setting. The values for all other positions were calculated from the wedge calibration.

When red light was used, the density of the Wratten No. 70 filter was subtracted from the log of the brightness with white light without filters. The known densities of the various neutral filters in red light were used in preparing a table of brightnesses for the various filter combinations. The ΔI field was then matched against the I field

2. *Procedure.*—In the experiments with white light $\Delta I/I$ was measured over a range of low to moderate brightness, a single test object being used for each sitting. There were two observers. One, J.R., had a Snellen acuity of better than 20/20. The other, M.A., used a lens inserted at the eyepiece to correct for myopia and astigmatism. Before each experiment the subject was dark-adapted for at least 30 minutes; measurements were then begun at the lowest possible brightness. In subsequent measurements the brightness was increased, in steps of 0.3 to 1 log unit, until a brightness of 10 millilamberts was reached. Because of the fixed 2 mm. pupil this gives a retinal illumination equal to that given when a surface of about 2.2 millilamberts brightness is viewed with the natural pupil. A complete set of data was obtained in from 1 to 2 hours. Since the work was arduous and tedious, frequent rests were given, and only one sitting was scheduled per day.

Thresholds were determined by a modified form of the frequency of seeing method described by Hecht, Shlaer, and Pirenne (1942). The observer was first presented with an easily visible ΔI field; the experimenter then moved the wedge so that succeeding exposures decreased in brightness, until the ΔI field became invisible. With the threshold thus roughly determined, three or four different values of ΔI were chosen for further investigation. These brightnesses were 0.24 log unit apart (1 cm. on the wedge) and covered the range just above and below the threshold. Five exposures of the ΔI field were given at each brightness, in random order of brightness and position. The subject was then given a short rest, followed by a repetition of the procedure. Occasionally there was a marked difference between the two groups of measurements; more exposures were then given. Sometimes the range of brightnesses chosen proved inadequate and an additional brightness step was added at the top or the bottom of the range. In this way a series of ten or more measurements at each of three to five different brightnesses was made, and the frequency of seeing at each brightness determined. These values were corrected for guessing, then plotted on a graph with log ΔI on the abscissa and the frequency of seeing on the ordinate. A smooth curve was drawn through the points, and the value of ΔI at which it crossed the 60 per cent seeing line was chosen as the threshold.

Since guessing was permitted, it had to be taken into account in the scoring. The target might appear in any one of the four positions; therefore its position would be reported correctly in 25 per cent of the presentations, even if it were not seen at all, simply on the basis of chance. Thus it was assumed that for every three incorrect responses there was one correct response as a result of guessing. To determine the number actually seen in a given set of responses, the number wrong was divided by three, and the quotient subtracted from the number correct.

In the second series of experiments a red filter was inserted at the eyepiece. This was a Wratten No. 70 filter, whose transmission below 650 mμ is practically zero. By this means the investigation was limited to cone vision only. This is because the foveal cones are more sensitive to red light than the rods in any region of the retina (Wald, 1945). There were two new subjects in this series; both had normal visual acuity.

At first, thresholds were determined just as in the first series. Later the procedure was modified so that two thresholds could be determined at the same time. The lower threshold was that at which the ΔI field could merely be seen 60 per cent of the time; the higher one that at which its orientation could be correctly determined in 60 per

cent of the exposures. These may be called light threshold and form threshold. In this new procedure, the subject was asked to report the position of the test object if he could; to report "yes" if a formless light was seen; and to report "no" if no ΔI field was seen. Brightnesses of ΔI were chosen to range from one at which the position was seen in 90 per cent or more of the exposures, down to one at which light was seen in 10 per cent or less.

The possibility that the subjects gave positive responses when they actually saw nothing was checked by giving exposures from time to time in which the ΔI field was completely occluded. Fortunately, "yes" responses occurred only rarely, and when there were more than one in a set of data, the threshold was redetermined at a time when the subject was more reliable.

TABLE I

$\Delta I/I$ as a Function of Brightness and Visual Angle, in White Light

Each datum is the average result from subjects M.A. and J.R. The target was rectangular, with a length-width ratio of 2. Visual angles are in minutes.

Background intensity log of I	Log contrast fraction, $\Delta I/I$							
	Log visual angle = 1.70	Log visual angle = 1.50	Log visual angle = 1.30	Log visual angle = 1.10	Log visual angle = 0.90	Log visual angle = 0.70	Log visual angle = 0.50	Log visual angle = 0.30
millilamberts								
$\bar{5}.12$	1.61							
$\bar{5}.69$	0.95		1.47					
$\bar{4}.13$	0.54	0.69						
$\bar{4}.46$	0.33		0.97		1.77			
$\bar{4}.66$		0.25		1.08				
$\bar{3}.13$	$\bar{1}.93$	0.09		0.68				
$\bar{3}.42$	$\bar{1}.69$	$\bar{1}.90$	0.20	0.50	1.08			
$\bar{3}.70$	$\bar{1}.52$			0.39	0.85			
$\bar{2}.08$	$\bar{1}.38$	$\bar{1}.64$	$\bar{1}.87$	0.19	0.53	0.99	1.19	
$\bar{2}.37$	$\bar{1}.35$	$\bar{1}.48$	$\bar{1}.81$	$\bar{1}.97$	0.36	0.69	1.00	
$\bar{2}.65$	1.34	$\bar{1}.35$	$\bar{1}.55$	$\bar{1}.78$	0.01	0.40	0.63	
$\bar{1}.10$	1.08	$\bar{1}.08$	$\bar{1}.34$	$\bar{1}.48$	$\bar{1}.70$	0.06	0.22	0.81
$\bar{1}.67$	$\bar{2}.70$	$\bar{2}.73$	$\bar{2}.75$	$\bar{1}.15$	$\bar{1}.34$	$\bar{1}.67$	$\bar{1}.72$	0.19
0.18								$\bar{1}.80$
0.75	$\bar{2}.21$	$\bar{2}.35$	$\bar{2}.42$	$\bar{2}.57$	$\bar{2}.82$	$\bar{2}.95$	$\bar{1}.08$	$\bar{1}.47$
1.79				$\bar{2}.43$				$\bar{1}.22$

III

Measurements

1. White Light.—The results for the two observers were so nearly alike that they were averaged. They are given in Table I and are plotted in Fig. 3. It will be seen that $\Delta I/I$ decreases as the size increases, and that the effect of size

becomes smaller when the largest sizes are reached. The characteristic break between rod and cone function is apparent with the larger targets. For the 8′ target the break is very slight, since rod and cone curves have very nearly the same slope at the transition point. For sizes smaller than 8′ there is no indication of rod function. This is in keeping with the subjects' reports that with the three smallest sizes their best discrimination was obtained with foveal fixation, even at the lowest brightnesses. Evidently, below the brightness at which the cones function, the rod system does not have the necessary resolving power to achieve a visual acuity of 0.2 (5′ visual angle).

The curves drawn through the data are theoretical, and will be discussed
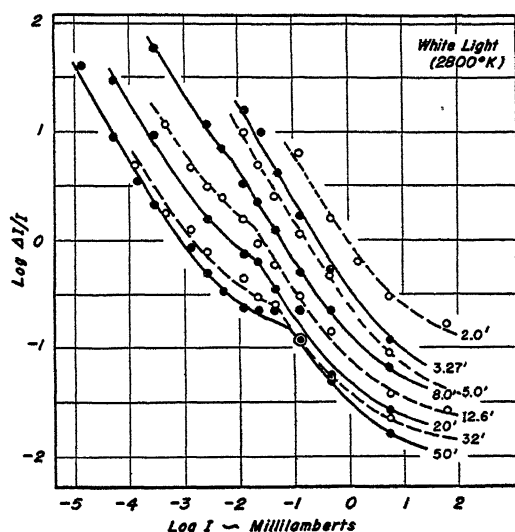
FIG. 3. Log $\Delta I/I$ as a function of log I, with a visual acuity criterion of seeing, for white light. The data of M.A. and J.R. have been averaged. The figures at the lower right are target widths, increasing from top to bottom in steps of 0.2 log unit. The curves are from equation (2).

later. The data are adequately described by the curves, though the fit is neither critical nor unique.

From the measurements in Fig. 3 one may derive curves relating brightness to visual acuity at fixed contrast. These are shown in Fig. 4. (The points were secured by reading across Fig. 3 at fixed levels of $\Delta I/I$, and recording the brightness at which the curves are intersected. Before these data were plotted, they were transformed into test object brightness ($\Delta I + I$), since at high values of $\Delta I/I$ the two differ widely, and it is the test object brightness that determines the visual performance.) The separation into rod and cone function appears at the higher values of $\Delta I/I$, while at the lower contrasts there is a single curve

representing cone function only. The rods cannot discriminate $\Delta I/I$ below about 0.22 (log $\Delta I/I = -0.65$).

In Fig. 4 the curves move down to lower visual acuity as the contrast is decreased. In addition, the cone curves move to the right, toward higher light requirements, though this is not obvious in Fig. 4. At any fixed target brightness, log visual acuity decreases at an accelerating rate, as log $\Delta I/I$ is lowered. For example, a log target brightness of 0 may be selected. Reducing the contrast from 10 to 1 lowers visual acuity by only 0.1 log unit. A further reduction in contrast of 1 log unit, however, lowers visual acuity by 0.56 log unit. At
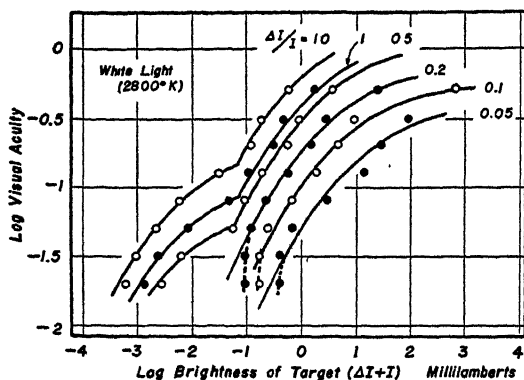
FIG. 4. Log visual acuity as a function of log target brightness, for white light. The data of M.A. and J.R. have been averaged. The values of $\Delta I/I$ are shown at the top. The continuous lines are from equation (3). The broken lines show where the data depart from these curves. The points were secured by reading across Fig. 3 at fixed levels of $\Delta I/I$, and recording the brightness at which the curves are intersected. Before these data were plotted, they were transformed into test object brightness ($\Delta I + I$), since at high values of $\Delta I/I$ the two differ widely, and it is the test object brightness that determines the visual performance.

lower brightness visual acuity falls off more rapidly as contrast is lowered. At a log target brightness of -1, for example, the visual acuity is lowered by 0.25 log unit when the contrast goes from 10 to 1. When contrast is further reduced by 1 log unit, the target becomes invisible at any size.

Fig. 4 shows that at the lowest visual acuities the data for low contrasts depart from the theoretical curves. The slope reaches infinity, whereas the theoretical curve has a maximum slope of 1. This discrepancy is to be expected, since we know that with increasing target size $\Delta I/I$ decreases to a minimum, where further increase in size has no effect. This point has probably been reached at the largest size in Fig. 3. Thus when $\Delta I/I$ is plotted against I for this largest target, the threshold brightness for any selected contrast may be read from the curve. If the brightness is lowered beyond this point, a target

of that particular contrast becomes invisible at any size. Thus curves such as those of Fig. 4 must become vertical at some brightness. It is of interest that Hecht and Wald (1934) found a minimum brightness at which the visual acuity curve of *Drosophila* became vertical.

2. *Red Light.*—The average results for two observers are given in Table II, and plotted in Fig. 5. The data have approximately the same form as those of Fig. 3 except that single curves fit the data, since the study was limited to cone vision only. The $\Delta I/I$ difference for each step in size becomes smaller as the size increases. This is strikingly true at the highest brightness, where the difference between 2' and 3.27' is as great as that between 5' and 50'.

TABLE II

$\Delta I/I$ *as a Function of Brightness and Visual Angle, in Red Light*

Each datum is the average result from subjects S.F. and G.G. The target was rectangular, with a length-width ratio of 2. Visual angles are in minutes.

Background intensity log of I	Log contrast fraction, $\Delta I/I$							
	Log visual angle = 1.70	Log visual angle = 1.50	Log visual angle = 1.30	Log visual angle = 1.10	Log visual angle = 0.90	Log visual angle = 0.70	Log visual angle = 0.50	Log visual angle = 0.30
millilamberts								
$\bar{2}.63$			$\bar{1}.88$					
$\bar{2}.93$	$\bar{1}.37$		$\bar{1}.59$		0.05	0.34	0.65	1.16
$\bar{1}.25$			$\bar{1}.33$		$\bar{1}.75$			
$\bar{1}.55$			$\bar{1}.10$					
$\bar{1}.86$	$\bar{2}.75$		$\bar{2}.88$		$\bar{1}.24$	$\bar{1}.60$	$\bar{1}.83$	0.63
0.88	$\bar{2}.44$	$\bar{2}.55$	$\bar{2}.49$	$\bar{2}.60$	$\bar{2}.78$	$\bar{1}.00$	$\bar{1}.24$	$\bar{1}.78$
1.91	$\bar{2}.26$	$\bar{2}.40$	$\bar{2}.40$	$\bar{2}.53$	$\bar{2}.63$	$\bar{2}.75$	$\bar{1}.07$	$\bar{1}.39$
3.37	$\bar{2}.33$	$\bar{2}.39$	$\bar{2}.33$	$\bar{2}.33$	$\bar{2}.49$	$\bar{2}.61$	$\bar{2}.89$	$\bar{1}.20$

Visual acuity at selected values of $\Delta I/I$ is plotted as a function of test object brightness in Fig. 6. These data clearly depart from the theoretical curves at the low brightness end. The trend of the data is toward a slope of infinity.

IV

Theoretical Curves

The theoretical curves in Figs. 3 to 6 come from the stationary state equation developed by Hecht (1934). This equation represents the most general requirements of a photoreceptor process, in which a photosensitive material is bleached by light and reformed by a thermal reaction in which some of its products are involved. The equation is

$$KI = \frac{x^n}{(a - x)^m} \tag{1}$$

where I is the brightness of the light, $(a-x)$ and x are the concentrations of sensitive substance and photoproducts respectively, m and n are the orders of the
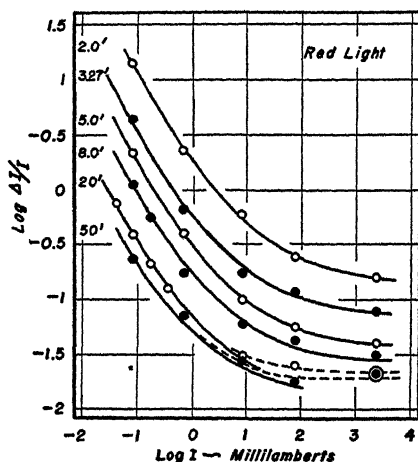
FIG. 5. Log $\Delta I/I$ as a function of I, with a visual acuity criterion of seeing, in red light. The data of S.F. and G.G. have been averaged. The figures at the upper left are target widths. The continuous lines are from equation (2). The dashed lines show where the data depart from these curves.
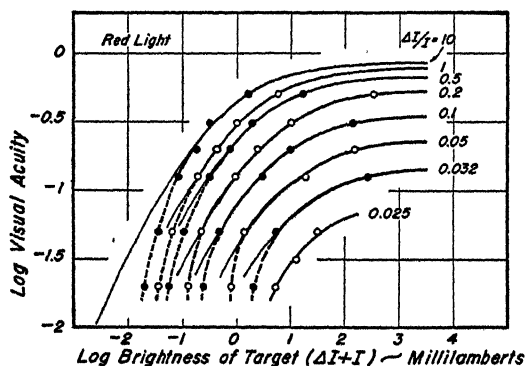
FIG. 6. Log visual acuity as a function of log target brightness, in red light. The data of S.F. and G.G. have been averaged. $\Delta I/I$ values are shown at the right. The continuous lines are from equation (3). The broken lines show where the data depart from these curves.

photochemical and thermal reactions, and K is the ratio of the velocity constants of the two reactions.

Hecht (1935) has shown how the equations for $\Delta I/I$ may be derived from equation (1). The only further assumption made is that the recognition of the

difference between I and $I + \Delta I$ is made immediately after ΔI is added and is determined by the initial rate of formation of photoproducts when ΔI is added. This added rate is assumed to be constant at all brightnesses, and means that in a given time the same increase in photochemical transformation is produced. On this basis, the equation for $\Delta I/I$ becomes

$$\Delta I/I = C \left[1 + \frac{1}{(KI)^{\frac{1}{2}}} \right]^{2} \tag{2}$$

when $m = n = 2$.

These values of m and n were chosen because many careful measurements of $\Delta I/I$ as a function of brightness have been made, and the data for human subjects follow equation (2), (Hecht, 1935; Hecht, Peskin, and Patt, 1938; Graham and Kemp, 1938; McFarland, Halperin, and Niven, 1944). Red light does not affect the shape of the curve, as shown by the data of Koenig and Brodhun (1888) and by Hecht, Peskin, and Patt.

In Figs. 3 and 5 the curve for equation (2) has been drawn through both the rod and cone portions of the data. The smaller targets are adequately described by this equation, but Fig. 5 shows that the largest sizes are fitted by the theoretical curves only at low brightnesses. The data show a much more rapid approach to a minimum $\Delta I/I$. At high brightness, some factor not considered in the theory seems to set a lower limit on the value of $\Delta I/I$ which cannot be passed regardless of size.

To make sure that this phenomenon was real, a number of additional measurements were made with the larger test objects, in both red and white light. The results with the 20' by 40' target are summarized in Fig. 7. The data clearly depart from the theoretical curves at brightnesses above 10 millilamberts, just as the earlier data did. Although in the experiments of Lamar et al. the criterion of seeing differed from that used in obtaining the other points in Fig. 7, the data from the two experiments seem to be comparable.

The curves of Figs. 4 and 6, where visual acuity is plotted as a function of brightness, are also derived from equation (1). Visual acuity is taken as proportional to x^{n}; hence the visual angle α varies as $1/x^{n}$. When $m = n = 2$ the equation may be put in the same form as equation (2), and becomes

$$\alpha = C \left[1 + \frac{1}{(KI)^{\frac{1}{2}}} \right]^{2} \tag{3}$$

Shlaer (1937) and Shlaer, Smith, and Chase (1942) have shown that this equation describes very well their precise measurements of the relation between visual acuity and illumination, with a Landolt ring as test object. The classic data of Koenig (1897) are also well fitted by equation (3). However, when the contrast between target and background is low, the theoretical curves do not fit at the low brightness end, for the reasons given in the discussion of Fig. 4.

V

The Distribution of Retinal Receptors

A possible explanation for the departure of some of the data from the curve based on a simple photochemical theory lies in the change in distribution of the functional retinal receptors with brightness.

It has long been known that the cones are most closely packed at the optical center of the eye, and that their population density falls off sharply toward the
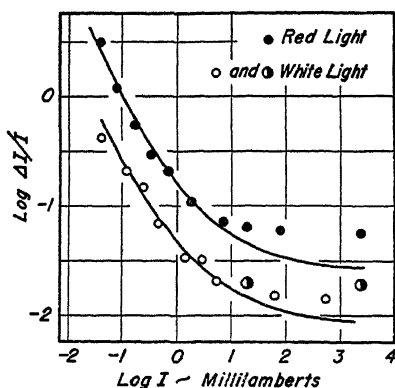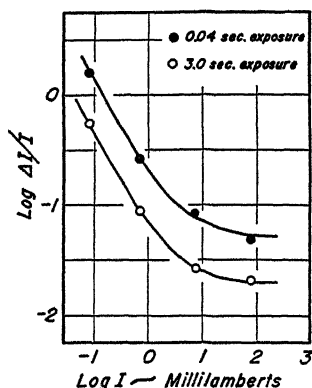
FIG. 7.   FIG. 8.

FIG. 7. $\Delta I/I$ as a function of I, for a 20' by 40' target. The filled circles show the average data of M.A., S.F., and C.H. in red light. These data have been raised 0.5 log unit on the ordinate. The open circles show the averaged data of M.A. and C.H. in white light. The half-filled circles show the average of five observers for white light from Lamar, Hecht, Shlaer, and Hendley (1947). The curves are from equation (2).

FIG. 8. $\Delta I/I$ as a function of I for exposures of 0.04 second and 3 seconds, 20' by 40' target. Red light was used. The data of subjects Y.H., D.H., and S.M. have been averaged.

periphery (Schultze, 1866). However, it is often tacitly assumed that their distribution is homogeneous in the 2° rod-free central region ordinarily called the fovea. Several authors have shown that it is precisely in this region that the distance between adjacent cone centers changes most rapidly (Rochon-Duvigneaud, 1906–07; Østerberg, 1935; Polyak, 1941). According to Polyak the thinnest cones are at the very center of the fovea, where they may be as small as 12″ or 15″ of arc. Around the center is a region of about 10′ radius containing some 2000 closely packed cones averaging 24″ in diameter. This region may be called the foveola. The distance between adjacent cone centers increases to 40″ at the edge of the fovea, about 1° from the center. At 3° 20′, in the parafoveal region, this distance is 80″. The cones become more and more

widely separated at greater distances from the center until in the far periphery the distance between cone centers is over 180″.

The fineness of the retinal grain has, of course, a profound effect upon visual function. It is well known that visual acuity is at its best at the center of the fovea, provided the illumination is adequate for the functioning of that region. It is also true that minimal values of $\Delta I/I$ are achieved only in the fovea, and probably only in the foveola. No matter how large the ΔI field is, the subject fixates one edge of it in order to see it when its contrast is at a minimum. Furthermore, a comparatively small target centrally fixated is visible at lower contrast than a much larger one whose inner edge is 1.25° away from the center of the eye (Lamar et al.).

Thus far we have been considering the situation at high illumination. When the brightness falls below the level that yields optimal visual performance, the superiority of the foveola over the rest of the fovea in the recognition of brightness differences begins to diminish. Visual acuity also tends to become more uniform over the fovea.

A probable explanation of this phenomenon arises from the fact that the regions in which larger cones are found are more sensitive than the foveola, with its extremely attenuated cones. At low brightness the foveola loses its fineness of grain, as an increasing fraction of the receptors fail to function with decreasing brightness. This occurs to a lesser extent further out in the fovea, with the result that a point is reached at which the foveola has a coarser grain than the rest of the fovea.

The effect of size on the shape of the curves of Fig. 5 may be explained on the basis of this change in the retinal mosaic with illumination. Considering first low brightness, we know that $\Delta I/I$ decreases as the size increases, though at a decreasing rate. The 50′ by 100′ target yields a lower $\Delta I/I$ than the 20′ by 40′ one. On the basis of the ideas outlined above this may be explained by assuming an approximately uniform distribution over the foveal region of the capacity for discrimination. Thus the increase in width from 20′ to 50′ brings into play new receptor areas, equally sensitive to those originally involved, and the threshold is consequently lowered.

At the highest brightness, however, the situation is quite different. A target 13′ by 26′ gives just as low a threshold as any of the larger sizes. Further increase in size produces no diminution of the threshold because the additional area falls on a retinal region whose grain is too coarse to contribute to the discrimination.

It is now clear why size affects the shape of the curves in Fig. 5. The theoretical curves fit the smaller sizes, where the retinal mosaic is reasonably uniform at all brightnesses. At the larger sizes, the effective area decreases as the brightness increases. Consequently the values of $\Delta I/I$ at high brightness are pushed up and the curve rapidly approaches a minimum with increasing brightness.

VI

The Effect of Exposure Time on the Relation between $\Delta I/I$ and Brightness

In these experiments, the target was exposed for 3 seconds in each presentation. This procedure allowed ample time for the subject to give his best performance, and approximated certain practical conditions of seeing that this work was designed to investigate. However, a possible complication was introduced by choosing this relatively long exposure time. For short exposures time t and intensity I are reciprocally related. With increasing time a minimal intensity is reached which remains constant with further increase in time. The time at which the transition occurs from the relationship $It = C$ to $I = C$ is called the "critical time" or the "retinal action time."

The critical time for several visual functions has been found to be much longer at low brightness than at high. It may vary from 0.1 or 0.2 second down to about 0.04 second. Graham and Kemp (1938) have pointed out that the curve relating $\Delta I/I$ to brightness will have one shape when the exposure is below the critical time at all brightnesses, and another when the critical time is exceeded at all brightnesses. For if a fixed brightness is chosen and the duration of exposure is increased, the threshold falls until the critical time is reached, when it becomes constant. Since the critical time is reached sooner at high brightness than at low, there will be less lowering of the threshold when the exposure is increased beyond the critical time.

In the present work, the shape of the curves of $\Delta I/I$ against I changes with size rather than exposure time. However, there remains the possibility that the relation between critical time and brightness may be dependent on target size. To test this hypothesis, measurements were made comparing a 0.04 second exposure with the 3 second one, for the 20' by 40' target. Red light was used, and the significant range of cone brightness was covered. At each brightness, successive measurements at the two different times were made. The results are shown in Fig. 8 where four experiments with three different subjects have been averaged. The same curve has been drawn through the two sets of data, which are displaced 0.45 log unit on the $\Delta I/I$ axis. There is no significant effect of exposure time on the shape of the curves, though the data are too scanty to establish that there is no effect at all. Steinhardt (1936) and Smith (1936) showed that their data were fitted by Hecht's equation, though the exposure was continuous. These results indicate that, under some experimental conditions, the critical time does not vary continuously with brightness.

VII

Visual Acuity as a Function of $\Delta I/I$

The data of Tables I and II may be presented so that visual acuity is a function of $\Delta I/I$. The necessary data were derived mainly from the curves of Figs.

4 and 6, by drawing vertical lines at selected brightnesses, and reading off the acuity values at which the curves were intersected. Some supplementary points were obtained from Figs. 3 and 5, by transforming the abscissa into a scale of log $(\Delta I + I)$, and reading off the $\Delta I/I$ values for the various sizes at a selected log $(\Delta I + I)$.

The results are shown in Fig. 9. The curve for a brightness of 10 millilamberts is very similar to those at higher levels of illumination. The 0.2 millilambert curve is near the lower limit for cone vision in white light. At 0.01 millilambert only the rods function, therefore there are no data here for the red light series. All the curves have the same general form, approaching a maximum visual acuity as the contrast becomes very great, and approaching a minimum value of $\Delta I/I$
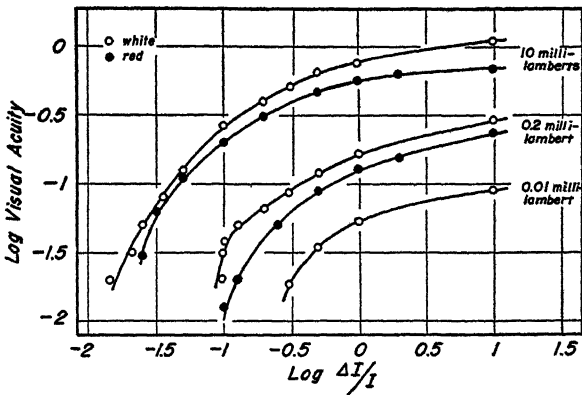
FIG. 9. The relation between log visual acuity and log $\Delta I/I$. The target brightnesses are shown on the right. The open circles are the data in white light of subjects J. R. and M.A.; the filled circles are the red light data of S.F. and G.G. The curves are empirical.

as the size becomes large. The red and white light curves are nearly parallel. At the higher brightness, the red light data show a more rapid approach to a constant $\Delta I/I$ value, while at the lower brightness the reverse is true. This difference is believed to be fortuitous, because of the peculiar relationship between the cone curves for the two largest sizes in Fig. 3. The fact that lower acuities were found in red light than in white may be ascribed to differences between the two groups of subjects.

It is apparent that visual acuity bears no simple relationship to $\Delta I/I$. As the brightness decreases, the point at which the curves become vertical moves to the right, since the $\Delta I/I$ threshold increases. The contrast at which the visual acuity reaches a maximum shows no appreciable change. This means that the form of the curves cannot be invariant since they change slope more abruptly as the brightness is decreased. The data may be fitted by hyperbolas of the

form $y = (x/a + bx) + c$, but different constants are required for each brightness.

These results agree with other work that covers a wide contrast range (cf. Cobb and Moss, 1928). Other authors have found visual acuity to be proportional to the square root, the first power, or the square of the contrast. These equations give straight lines that fit the data of Fig. 9 only over short ranges of contrast.

VIII

Frequency of Seeing

1. *Theory.*—The thresholds reported in this paper were determined by measuring the frequency of seeing as a function of $\log \Delta I / I$. Hecht, Shlaer, and Pirenne (1942) have shown how such data may be used to determine the minimum number of retinal events necessary for seeing in the dark-adapted eye. Their ideas will be briefly summarized here.

A flash of light will be seen against a background of total darkness when more than a certain minimum number of light quanta are absorbed by the photoreceptor cells. Since these quantum absorptions are discrete, independent, and random events, the number in any given flash of light will vary according to a Poisson probability distribution (Fry, 1928). If a is the average number of quanta absorbed per flash, the probability P_n that any number n will be absorbed is given by the formula

$$P_n = \frac{a^n e^{-a}}{n!} \qquad (4)$$

where e is the base of natural logarithms. If one assumes that the light is seen when N or more quanta are absorbed, the integral of equation (4) from $n = N$ to $n = \infty$ gives the probability of seeing. In Fig. 10 some of these Poisson integral curves are shown.

The abscissas in Fig. 10 are expressed as the logarithm of the average number of events. Experimental data may therefore be compared with Fig. 10 by plotting the frequency of seeing against the logarithm of the brightness, since the average number of quanta absorbed by the retina is proportional to the brightness. By fitting the experimental curve with one of the probability distributions in Fig. 10 the number of events involved in a visual discrimination can be determined.

Hecht, Shlaer, and Pirenne concluded that "the number of critical events in the retina required to produce a visual effect lies between 5 and 8." This agreed very well with the value of 5 to 14 quanta absorbed by the retina, obtained from physical measurements of the energy incident at the cornea, corrected for the transmission of the ocular media and the absorption of rhodopsin in the retina.

They concluded, therefore, that the critical retinal events were quantum absorptions.

The frequency of seeing curves found by Hecht, Shlaer, and Pirenne was obtained under conditions that reduced to a minimum the threshold number of quanta incident at the cornea. Other threshold measurements have been made using different field sizes, retinal locations, colors, and exposure times; yet the same number of critical retinal events is found. The data obtained with a 3° field in red and violet light are well fitted by the curve for $N = 6$ (Hendley and Frank, unpublished). The data of Hartline and McDonald (1943) for white light are fitted best when $N = 7$. Pirenne (1943) found that $N = 6$ for white light (cf. Pirenne, 1946). Peckham (1944) and Guild (see Stiles, 1944) obtained
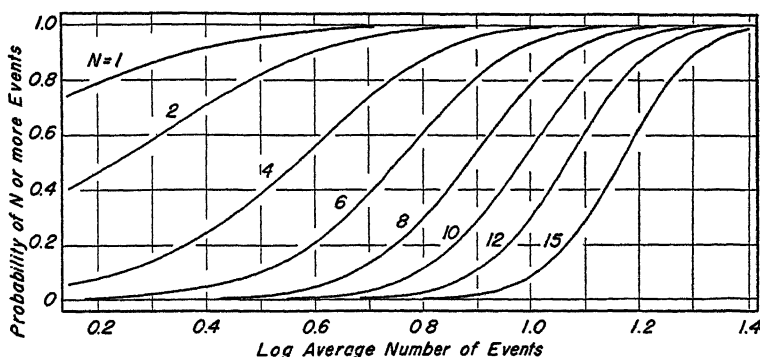
FIG. 10. Poisson probability integral curves. For any log average number of events, the ordinates give the probability of N or more events. The curves represent selected values of N from 1 to 15.

curves for $N = 6$ from the averages of very large numbers of threshold measurements.

These experiments show that although the threshold energy striking the retina varies enormously with wavelength, the threshold number of absorbed quanta does not change. Another important conclusion is that the size of the field does not influence the shape of the frequency of seeing curve, even though the threshold number of quantum absorptions increases when the area is larger thant 10'. This result may be explained by assuming that some of these absorbed quanta do not contribute to the production of a nervous discharge. Several hundred rod cells are connected to a single fiber in the optic nerve. It may be that about 6 quanta must be absorbed by the rods connected to a single fiber, in order to initiate a threshold visual response.

So far only measurements of the absolute threshold have been considered. The same reasoning may, however, be applied to brightness discrimination. When the eye views the background field it is in a photostationary state in

which a constant photochemical effect is producing a constant number of nerve impulses in unit time. If an additional amount of light (ΔI) is now added to part of the field there is an initial increase in the number of quanta absorbed, yielding additional nerve impulses from this region. It is possible that when the ΔI field is at the threshold of visibility, this number of quanta is the same as at the absolute threshold. In fact, Hecht's theory of brightness discrimination points toward this result. He proposed that the initial increase in rate of decomposition of sensitive material resulting from the addition of a threshold ΔI is the same at all background brightnesses. This means that the number of additional quanta absorbed is constant, since there is a direct relation between quantum absorption and decomposition. We know that this number must be about 6 at very low brightnesses, for as the background brightness approaches zero, the test situation becomes one in which the absolute threshold is measured. Presumably then, the number is the same at all higher background brightnesses and we should expect similar frequency of seeing curves.

2. *Experimental.*—To establish the thresholds given in Tables I and II we used a frequency of seeing method, as discussed in section II. Our interest was in establishing the 60 per cent seeing point, and we took only enough readings to set this. Usually there were ten exposures at each of four or five different brightnesses. This was insufficient to yield smooth frequency of seeing curves. If for example, the frequency of seeing at a given brightness is 10 per cent there will not always be just 1 positive response in a set of ten exposures. The probability is 0.6 that the datum obtained will deviate by at least 10 per cent from its true value.

In view of the irregularity of the experimental data, it was necessary to average a number of curves. This was done by drawing completely empirical smooth curves passing through all the experimental points. These curves were then averaged horizontally, by averaging the abscissas at which they crossed a fixed ordinate. The ordinates chosen were 95, 90, 80, 60, 40, 20, and 10 per cent seeing.

The data used for determining these composite frequency of seeing curves were those of observers S.F. and G.G. in red light. Only those experiments were considered in which both light and form thresholds were measured. The data at a single size and brightness were not numerous enough to yield smooth frequency of seeing curves. Therefore in grouping the data several sizes and brightnesses had to be lumped together. The sizes were combined into two groups; those up to a width of 5' as small sizes, the rest (8' to 50' in width) as large. The brightnesses were divided at 10 millilamberts into high and low brightness groups. These divisions were chosen to give groups of data of approximately equal size.

The high brightness data are shown in Fig. 11. They were well fitted by Poisson integral curves from Fig. 10. The value of N was 6 for the light thresh-

old, at all target sizes. The form threshold data were fitted when $N = 8$ for the small targets, and $N = 11$ for the large. The curves drawn through the data of Fig. 11 are, however, slightly modified Poisson integrals, with smaller values of N. This requires some explanation.

In the application of the Poisson theory to threshold measurements, it has been assumed that the eye has but one look at the target in a single exposure. In the present work, however, the subject was given 3 seconds to look for the target. If one knew just how many looks the subject had in each exposure, and if one could assume that there was an equal chance of seeing in each look,
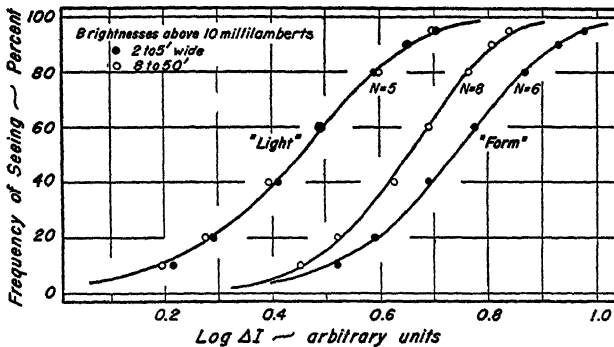
FIG. 11. Frequency of seeing curves at high brightnesses, of subjects S.F. and G.G. The light threshold data are at the left, the form threshold data at the right. The data for small sizes (filled circles) are the average of sixteen individual frequency of seeing curves. The data for large sizes (open circles) are the average of seventeen curves. The data have been shifted along the ΔI axis so that the light threshold is the same for all sizes. The curves are modified Poisson integrals for $r = 2$ (see text).

one could evaluate the effect on the frequency of seeing curve. From the definition of probability the following equation would hold:

$$P_r = 1 - (1 - p)^r \tag{5}$$

where p is the probability of seeing in a single look and P_r the probability of seeing at least once in r looks. Combining equation (5) with the integral of equation (4), the probability of N or more events occurring at least once in r looks may be determined. The effect of increasing the number of looks when $N = 6$ is shown in Fig. 12. Here the effect of changing the number of looks in an exposure is to move the frequency of seeing curve to the left on the intensity scale, and to make it steeper. Pirenne (1943) has shown how the difference between monocular and binocular absolute thresholds may be explained on this basis, since two eyes give two looks in a short exposure.

When there is a single 3 second exposure, however, the observer makes a single response as to whether he sees or not. If he gets only one fleeting glimpse of an

object in a long exposure, he may be influenced by his impression of *not* seeing
it most of the time, and make a report of not seen.   The subjects also reported
that they would almost always see the target right at the beginning of the ex-
posure or not at all.   Thus the assumption made above that there is an equal
chance of seeing in each look is almost surely false.

In view of this equivocal situation, some measurements were made in which
the thresholds for 0.2 second and 3 second exposures were compared.   A time of
0.2 second is longer than the critical time, yet short enough to give only one
look.   The threshold for the longer exposure was lower by 0.1 log unit.   This
is just the difference between the thresholds for one and two looks in Fig. 12,
where $N = 6$.   For other small values of N, the separation would be very nearly
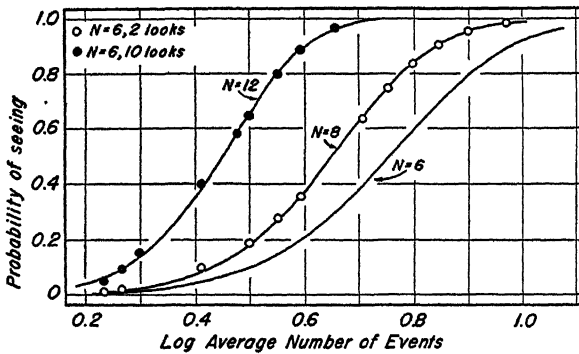
FIG. 12. The effect of increasing the number of looks per exposure on the shape of
frequency of seeing curves.   The curve at the right is the Poisson integral for $N = 6$.
The points are calculated from equation (5) and the integral of equation (4), when
$N = 6$.   The open and filled circles represent two and ten looks per exposure re-
spectively.   They are fitted by curves from Fig. 10.

the same.   The conclusion was drawn that there are effectively only about two
looks in a 3 second exposure.

Setting the value of r in equation (5) at 2, a new set of Poisson integral curves
was constructed, and these are the curves drawn through the data of Fig. 11.

In Fig. 13 the low brightness data are shown.   The curves drawn through the
data are again modified Poisson distributions for two looks per exposure.   Figs.
11 and 13 show that all the curves are of approximately the same shape, and all
are fitted by curves for small numbers of events.   The values of N vary from 4
to 8 when $r = 2$.   This is in good agreement with absolute threshold measure-
ments for which the average value of N was 6.   Therefore we may conclude that
there are no more than two effective looks in a single exposure.   For if r were
greater than 2, N would be less than 4 to 8.   We have no reason to believe that
the number of critical events involved in a differential threshold discrimination

would be any less than the number involved at the absolute threshold.   On the other hand, if the value of r is 1, the critical number of events varies from 5 to 11.

In Table III the values of N of the various curves are summarized.   There are no striking effects of size or brightness on the shape of the curves.   There is
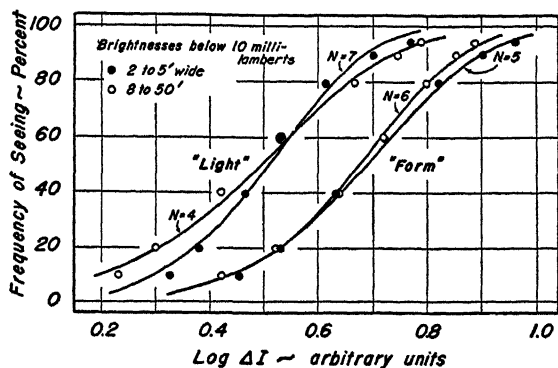
FIG. 13. Frequency of seeing curves, at low brightnesses, of subjects S.F. and G.G. The light threshold data are at the left, the form threshold data at the right.   Each set of points represents the average of fifteen individual frequency of seeing curves. The data for small sizes are shown by filled circles, the large sizes are represented by open circles.   The data have been shifted along the ΔI axis so that the light threshold is the same for all sizes.   The curves are modified Poisson integrals for $r = 2$ (see text).

TABLE III

Critical Number of Events for Form and Light Threshold

Sizes	Brightnesses	No. of effective quantum absorptions				Form threshold Light threshold
		$r = 1$		$r = 2$		
		Light	Form	Light	Form	
Small	High	6	8	5	6	1.90
"	Low	9	6	7	5	1.55 '
Large	High	6	11	5	8	1.60
"	Low	5	8	5	6	1.48

a tendency for larger size and higher brightness to yield a steeper curve, but the trend is not significant.   The form thresholds are, of course, higher than the light thresholds.   The difference is greater for the small targets than for the large, and greater at high brightness than at low.   These differences are small, and may not be significant.

A point of considerable interest is a comparison of the shapes of the curves for light and form threshold.   It might reasonably be assumed that if about 6

effective quantum absorptions are required for just seeing a test object, more will be necessary in order to see it clearly enough to determine its orientation, and the frequency of seeing curve will be steeper. Hartline and McDonald (1943) did find this to be true. It may be, however, that although more quanta must be absorbed at the form threshold, the number that are ineffective also increases, so that the number actually contributing to the discrimination remains the same.

The following hypothetical example may clarify this idea. The subject is viewing a background field of uniform brightness. The eye is in a steady state, with no sharp gradations in the rate of nervous discharge within that part of the retina covered by the background field. Then a ΔI field is added at the center of the fovea, and 6 additional quanta are absorbed from the retinal image in a short period of time. All those absorptions are effective in producing nervous discharges. They are, however, distributed over the whole area of the retinal image according to the degree of contrast in the different regions of the image. They are likely to be so scattered that the pattern of stimulation does not reproduce the contour of the test object accurately enough to determine its orientation.

If the number of the absorptions is doubled, the contour of the object is better defined, and its orientation can be determined. But some of these absorptions fall in the central region where they give no information about which is the long axis of the rectangle and which the short one. It is only those absorptions that occur near the ends of the long axis that are critical, and these determine the shape of the frequency of seeing curve. If six of the twelve absorptions are critical, we obtain a curve with the same slope as the light threshold.

The results of the present experiments are that in three out of the four pairs of curves the form threshold curve yields the higher value of N. For the smaller sizes at the lower brightnesses, however, the value of N is actually lower for the form than for the light threshold. Only further experiments can decide whether this is fortuitous, or the expression of a real phenomenon. At the present time, we can only say that the number of critical events is nearly the same, whether the criterion of seeing is the detection of light or the distinction of form.

SUMMARY

1. Visual acuity depends on the brightness contrast between test object and background; and conversely, brightness discrimination depends on the target size. Both functions vary with the brightness of the background. Measurements with rectangular targets of length-width ratio 2 were made over a range of sizes, contrasts, and brightnesses sufficient to determine the relations among these three variables. The rectangles were from 2′ to 50′ wide; the contrast fraction, $\Delta I/I$, ranged from 0.01 to 40; the background brightness varied from 0.0001 to 2500 millilamberts.

2. When $\Delta I/I$ or visual acuity is plotted as a function of brightness the data do, in general, follow Hecht's equation. The departure from a simple photochemical theory which the larger targets show is probably due to changes in the functional retinal mosaic with changing brightness.

3. In general also, the relation between visual acuity and brightness, at selected contrasts, fits Hecht's derivation. At low contrasts, as the brightness is reduced a point is reached at which the test object becomes invisible at any size.

4. No simple relation emerges from the data relating visual acuity to contrast, at set levels of illumination. Over only a very short range are visual acuity and contrast directly related. At high contrasts, visual acuity reaches a maximum, whereas at low visual acuity, $\Delta I/I$ reaches a minimum which cannot be passed regardless of size.

5. The shape of the curves relating $\Delta I/I$ to brightness is not significantly altered by changing the exposure time. There is some evidence to show that a 3 second exposure of the target is equivalent to two looks of 0.2 second each.

6. In all these studies the thresholds were determined by a frequency of seeing method, and the data have been considered in terms of a quantum theory of threshold seeing. It was found that a threshold response involves between four and eight independent critical events, which are largely independent of size, brightness, and criterion of seeing.

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CORRECTION

The authors wish to correct an error in the paper "The behavior of the nucleic acids during the early development of the sea urchin egg (*Arbacia*)" (*J. Gen. Physiol.*, 1947–48, **31,** 203). Owing to an oversight, the figures for the amounts of various P fractions in a single *Arbacia* egg have been erroneously expressed in $\gamma \times 10^{-3}$ units (Tables I and II, page 205; the last two lines of page 206). The figures should have been expressed in $\gamma \times 10^{-5}$ units.

Thus, the fertilized *Arbacia* egg contains an average of 20 $\gamma \times 10^{-5}$ ribonucleic acid P and 0.7 to 1 $\gamma \times 10^{-5}$ desoxyribonucleic acid P.

The authors are greatly indebted to Dr. A. E. Mirsky for having called their attention to this error.

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THE VISUAL FUNCTIONS OF THE COMPLETE COLORBLIND*

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I

INTRODUCTION

The purpose of studying the various visual functions of a completely color-blind individual is to secure information about the process in the normal eye. Earlier studies of the completely colorblind have shown them to be of two distinct types. The first of these possesses a low intensity mechanism and a high intensity mechanism much like the normal eye, corresponding most probably to rod vision and cone vision respectively. Visual acuity is high like the normal, and sensory distribution in the spectrum shows a different location for the low intensity and the high intensity mechanisms. Evidently in such cases something has gone wrong with the properties of the three cone mechanism, and color vision is absent although the cones are still active. A second type of complete colorblindness is one in which there is only one visibility curve, and that corresponds to the low intensity spectrum distribution in the normal. Visual acuity also is low, and there may or may not be a central blind spot. In all likelihood, in these cases, the cones either do not exist or are completely non-functional. It is a case of this sort on which we wish to report.

In previous descriptions of such cases, one, or at most two visual functions have been described by either the visibility curve or the relation of visual acuity to illumination (Uhthoff, 1886; Koenig, 1897). The particular significance of our present contribution is that we have studied several of the functions in some detail and have compared them with the behavior of normal eyes with the same apparatus and under similar circumstances.
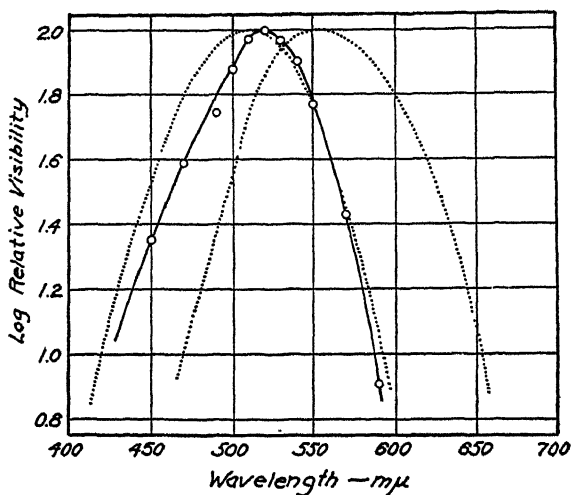
FIG. 1. Brightness distribution in the spectrum. The data for the normal eye are shown by the dotted curves: the one on the left for low intensity (rods) and on the right for high intensity (cones). The points and solid curve are for the complete colorblind.

II

Spectral Brightness Distribution

The measurements were made with the Helmholtz color mixer using the procedure and calibrations already described (Hecht and Shlaer, 1936). Fig. 1 shows the relative sensibility of our subject's eye in the spectrum at an ordinary high brightness; the data are given in Table I. Such a comparison is easy to make because the problem of heterochromic matching does not enter. A field of 1° was first tried, but the measurements were finally made with a 3° field because the subject was able to make more consistent brightness comparisons with a larger field. Included in the figure are first, the relative visibility curve for the human eye at low brightness as measured by Hecht and Williams (1922), and confirmed by Weaver (1937), and second, the bright visibility curve from the data of Gibson and Tyndall (1923). It is apparent that though made at a brightness which in the normal eye would yield the right-hand high visibility

curve, our subject shows a sensibility distribution in the spectrum corresponding to that found for rod vision at dim illuminations only.

Certain points are to be noted. The maximum of the standard scotopic visibility curve is at about 511 mμ. The maximum of our subject is more nearly at 520 mμ. Examination of the figure shows that the long wavelength portions of the two curves coincide almost perfectly, whereas they deviate on the short wave side. There are several factors which may explain this situation. The standard curve is an average of 48 individuals, and since these may vary somewhat in the precise location of the maximum, the average curve is slightly wider than that of any single individual. A more important factor

TABLE I

Brightness Distribution in Spectrum

The maximum is placed at 100. The measurements are the average of two runs made on successive days.

Wavelength	Relative brightness
mμ	
450	0.225
470	0.389
490	0.555
500	0.759
510	0.940
520	1.000
530	0.929
540	0.800
550	0.589
570	0.270
590	0.080

is the relative amount of pigment which absorbs light in the short wave portion of the spectrum. Keilin and Smith (1939) found two diffuse absorption bands at about 495 mμ and 455 mμ which were visible to the eye in a small dispersion spectrum. They also deduced the presence of pigment in the optical path of the eye from an examination of the visibility data for the normal and for the colorblind eye. These data reveal two distinct depressions in the cone visibility curves at the same wavelengths where the absorption bands can be seen directly. The visibility data of our complete colorblind also show a depression in the curve at 490 mμ. Since the measurements with our subject were made with central fixation, it is quite likely that the narrow shape of the curve of the colorblind is due in part to the yellow pigment of the macula lutea. The rod visibility curve of the normal individual is measured in the periphery of the eye, therefore, the macular pigment would not influence the curve. Wald (1945) has recently extracted the macular pigment of the human eye and found that it

possessed an absorption spectrum similar to leaf xanthophyll. He has also
shown that the difference in spectral sensitivity between peripheral and central
cones is due to the presence of the macular pigment in the center of the eye.
Still another factor may also influence the shape of the visibility curve and that
is the absorption by yellow pigment in the lens and ocular media of the eye
(Ludwigh and McCarthy, 1938).

Whatever the exact meaning of the minor deviations, it is reasonably clear
that our colorblind subject has elements in the retina which correspond fairly
well with our rods, and not at all with our cones.
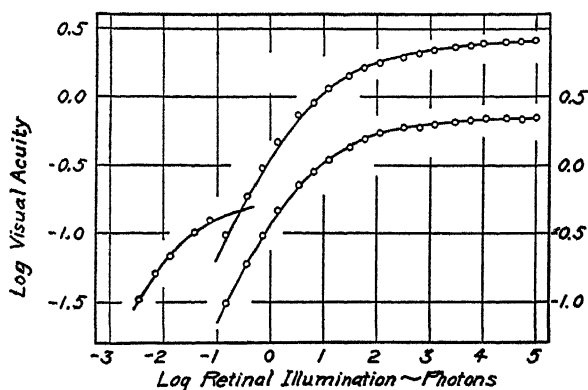
FIG. 2. Visual acuity for the normal eye. The upper curve shows two sections,
the one at low intensities made with the periphery of the eye, and the one at higher
intensities made with the rod-free center. The lower curve shows the data obtained
with the center of the eye at all intensities (ordinate on the right).

III

Visual Acuity

Koenig (1897) once measured visual acuity as a function of intensity with a
completely colorblind person and found that the relationship between these
two variables was much like that found for the normal eye at low illuminations,
although it extended beyond the normal rod region. Our meaning will be clear
from examination of Fig. 2 which shows this relationship for the normal eye
from the work of Shlaer (1937). The upper curve shows clearly that there
are two sections to the relationship between visual acuity and illumination,
one at low intensities which is measured with the periphery of the eye, and one
at higher intensities for which the measurements are made with the rod-free
center. The lower curve of Fig. 2 shows the data obtained with the rod-free
center of the eye, even at the lowest intensities visible.

If our subject possesses elements which have the characteristics of rods, the
high intensity section of Fig. 2 should not be present, but the low intensity

curve should extend into the high illumination region without much increase in visual acuity.   Fig. 3 presents the measurements made with our subject; the
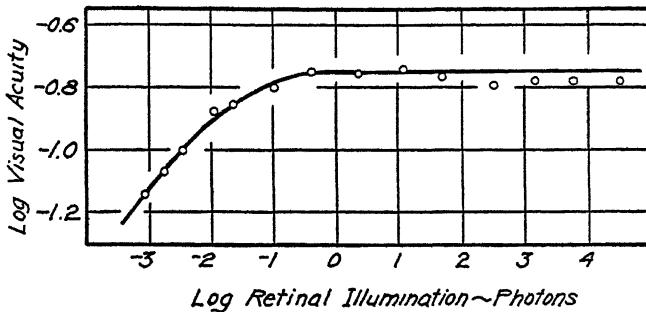
FIG. 3. Visual acuity for the complete colorblind eye.   Only a single curve is found and this corresponds to rod function.   The maximal visual acuity is only about one-tenth that found for cone vision in the normal eye.

TABLE II

The measurements were made with a 3 mm. pupil using blue light obtained with Wratten filter No. 75 and Corning filter No. 428; the dominant wavelength is 490 mμ. The log I values are from Table II of Shlaer, Smith, and Chase (1942), and have been corrected for the change in pupil size and the absence of the Stiles and Crawford effect for rod vision.

Log I in photons	Log visual acuity
−3.040	−1.142
−2.732	−1.067
−2.433	−1.000
−1.917	−0.875
−1.609	−0.853
−0.965	−0.797
−0.358	−0.747
0.387	−0.751
1.111	−0.737
1.719	−0.762
2.543	−0.789
3.187	−0.776
3.794	−0.776
4.539	−0.778

data are given in Table II.   It is apparent that there is only one relationship between visual acuity and illumination and that this corresponds very clearly to the rod function of the normal eye.   The measurements were made using the apparatus described by Shlaer (1937), employing as a test object a grating of alternate opaque and transparent bars of equal width.

IV

Dark Adaptation

The two functions of the colorblind eye just enumerated conform precisely to what is to be expected from previous knowledge and theory of normal vision. The next measurements show that there are some additional complexities, and
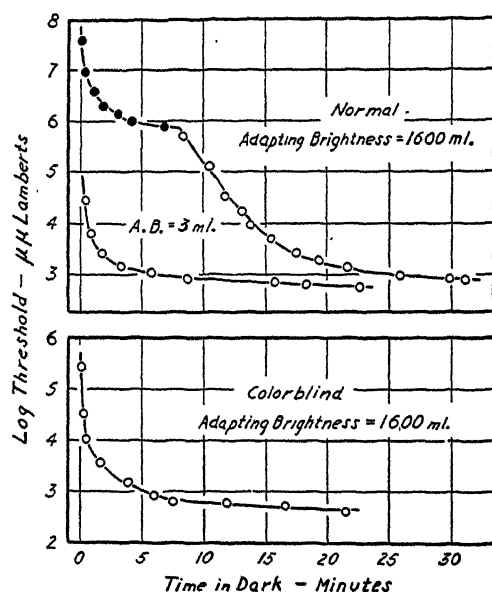
FIG. 4. Dark adaptation for the normal individual and for the complete colorblind. After adaptation to 1600 millilamberts, the normal shows a rapid cone adaptation (solid circles) followed by a slow rod adaptation (open circles). The colorblind shows only a rod curve of the rapid type. The threshold measurements were made with violet light (450 mμ) using a 3° field, 7° off-center (nasal) after preadaptation for 4 minutes. The data are averages of three runs made with the adaptometer described by Hecht and Shlaer (1938).

these may be introduced by examining the dark adaptation of normal eyes and of our subject's eye. The upper half of Fig. 4 shows normal dark adaptation under two conditions. The upper curve records what happens after light adaptation to 1600 millilamberts, and the lower one to 3 millilamberts. The dark adaptation following preadaptation to high brightness shows two sections; the first is cone adaptation and the second is rod adaptation. Following pre-adaptation to a low intensity, one finds in the normal eye only rod adaptation. Its shape, however, is different from the rod adaptation which follows high preadaptation because it is much faster, and instead of gradually leaving the

intensity axis, it does so sharply (Wald and Clark, 1937; Hecht, Haig, and Chase, 1937).

We had supposed that with our colorblind subject, following adaptation to high brightness, we should not find the preliminary cone adaptation but should
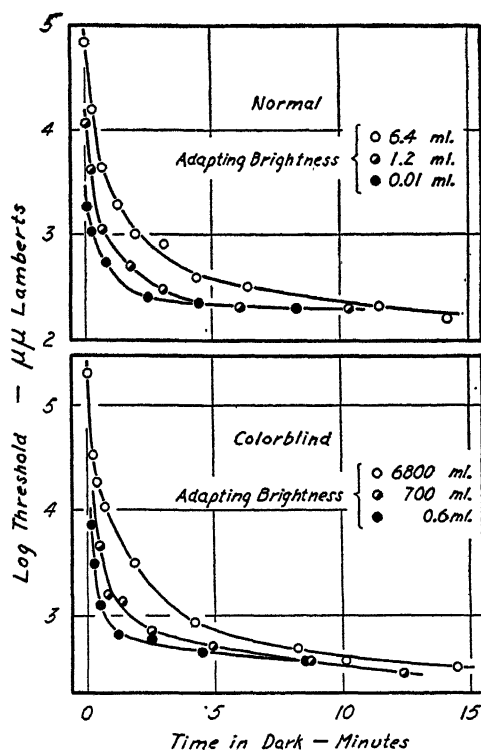
FIG. 5. Dark adaptation for normal and for the colorblind after different adapting brightnesses. The colorblind shows only rapid rod adaptation regardless of the intensity of the preadaptation. The measurements were made under the same conditions as those described in Fig. 4. However, similar results for the colorblind were also obtained using a 5° central field (violet) and a 3° central field (red).

immediately go to the subsequent rod adaptation, and thus secure information about that part of the rod adaptation curve which one can never obtain in normal eyes because of the speed and dominance of cone adaptation. The lower half of Fig. 4 shows the results secured with the colorblind subject. Although adapted to 1600 millilamberts, her eye showed a dark adaptation curve much more similar to the normal following low preadaptation than to the normal following high preadaptation. This situation is brought out more clearly in Fig. 5. The precise shape of the dark adaptation curve may be

varied by the intensity of preadaptation. The upper half of Fig. 5 shows for the normal eye the dark adaptation following three low intensity preadaptations; the lower half shows results with the colorblind. Approximately the same results as those with the normal are secured with intensities very much higher. In other words, under all circumstances, a completely colorblind subject shows only the fast type of rod dark adaptation.

Precisely what this means is hard to say at the moment because the nature and kinetics of even normal dark adaptation are not completely clear. The type of curve secured resembles more nearly what is to be expected in terms of a simple equilibrium between visual purple and its photoproducts, and the regeneration from them in the dark.

Table III gives the data for the threshold of the colorblind eye for different retinal positions. The data show that the threshold is about 1 log unit higher in the center of the eye than it is in the periphery. Haig and Haig (1947) have recently reported that for the normal eye, under identical conditions of measurement the difference in threshold between the rod-free center and the periphery is about 3 log units, while the difference in threshold between 2° off-center and the periphery is about 1 log unit. Thus the rod picture in our colorblind subject is the same as in the normal except for the presence in the center of rods having the same threshold as those 2° off-center.

V

Critical Fusion Frequency and Intensity Discrimination

The most striking thing which appeared in the measurements with our colorblind subject is in the data of flicker and of intensity discrimination. Fig. 6 shows the data secured for the relationship between intensity and the critical fusion frequency.[1] We used a large field and a small field but the results are much the same. For the normal eye, there is a marked increase in the rod curve when a large field is used as compared with a small one (Hecht and Smith, 1936). With the colorblind eye, there is no effect of field size. There seem to be two types of sensory systems in this eye, one functional at low illuminations, and the other at high illuminations. Naturally, one would immediately suspect that the high intensity sections represent cone function of perhaps a rudimentary kind. This, however, is not the case because the

[1] The intensity scale used for the different colors is that derived by heterochromic comparison with white light for the cone vision of the normal eye. Obviously, this has no real meaning for the colorblind eye which possesses a different sensibility distribution in the spectrum. The data have been left in this form for comparison with the normal eye. Logically, one should use the intensity comparison made by the colorblind. When this is done, the three curves of Fig. 6 show a transition between the two different functions at the identical intensity. This is also found for the intensity discrimination data of Fig. 8.

sensibility distribution of the high intensity elements is identical with that of the low intensity elements, as can be seen from Fig. 6 where the measurements

TABLE III

Threshold and Retinal Position

The measurements were made after complete dark adaptation with a 1° field using the light transmitted by Wratten filter No. 76 and Corning filter No. 428 (the color is violet with the dominant wavelength 450 mμ).

Distance from fovea	Log I in micromicrolamberts
degrees	
0	3.65
2.0	3.63
5.0	3.15
7.0	2.78
10.0	2.78
12.5	2.60
15.0	2.73
20.0	2.63
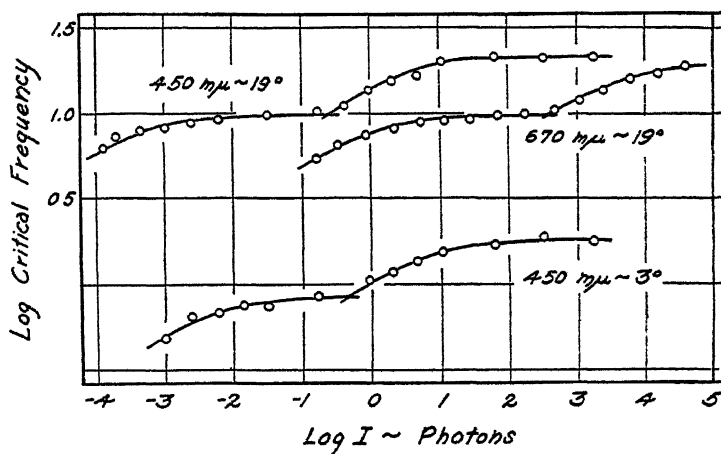
FIG. 6. Critical fusion frequency and intensity for the colorblind. Low intensity and high intensity functions are present but these are not influenced by the size of the field or the color.

are made with extreme violet and extreme red light. What may be expected when the two elements have different sensibility distributions in the spectrum is shown in Fig. 7 which records the behavior of the normal eye under similar circumstances (Hecht and Shlaer, 1936). Because the rods and cones have such different sensibilities in the spectrum, the measurements with red light show only cone function and not a trace of rod function, while with blue light

the measurements show a very large rod function because of the relatively greater sensibility of the rods in the blue. No such difference between red and blue is apparent in Fig. 6 which records the data of the colorblind. The measurements with the colorblind were made with the apparatus previously described (Hecht and Shlaer, 1936). The data are recorded in Table IV.

As Fig. 8 shows, the same phenomenon appears when we measure the intensity discrimination of the colorblind. Here also, there seem to be two systems, one functional at low intensities, and the other at high intensities. However, here too the measurements with blue light and red light show exactly
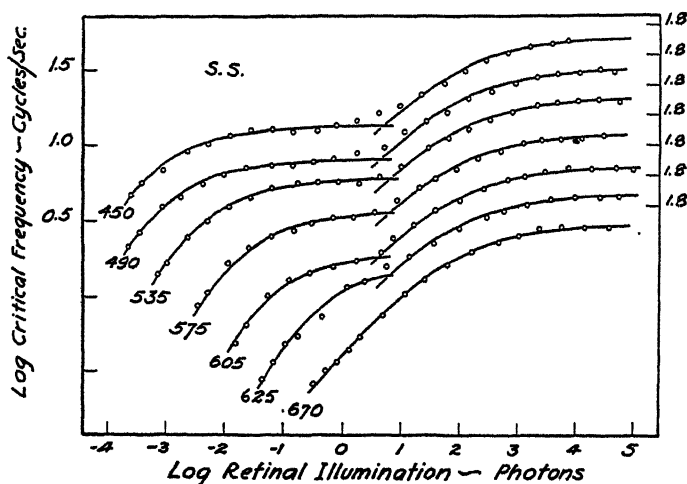
FIG. 7. Critical fusion frequency and intensity for different spectral regions for the normal eye. The measurements with red light show only cone function; those with other colors show both rod and cone function.

the same amounts of the two functions. Fig. 9 (Hecht, Peskin, and Patt, 1938) illustrates what happens in the normal eye under similar circumstances. It can be seen that with blue light there is a large rod section which is completely missing from the measurements made with red light.

It is noteworthy that the best intensity discrimination of the colorblind is very nearly the same as for the normal eye. This is in contrast to the data for critical fusion where the frequency for the normal is about 55 cycles per second as compared to 20 for the colorblind.

We must therefore conclude from the measurements of critical fusion frequency and intensity discrimination that although the colorblind eye possesses two systems functioning in different parts of the intensity range, these two systems possess sensibility distributions in the spectrum which are identical. It thus appears as though there are two kinds of rods present in the eye of our

colorblind. Although they possess the same sensitive material, namely visual purple, they have different intensity thresholds. We may account for this by supposing that the concentrations of visual purple are different in the two systems. Those rods which have the usual concentration of visual purple behave like ordinary rods, and are sensitive to the very lowest intensities as in the normal eye. Those rods, however, which contain visual purple in much lower concentrations would then have a threshold very much higher and would

TABLE IV

Critical Fusion Frequency and Illumination

Each set of data is the average of three identical runs. The measurements were made with central fixation.

Blue light (λ = 450 mμ)			Red light (λ = 670 mμ)	
Log I in photons	Cycles per sec.		Log I in photons	Cycles per sec. 19° field
	3° field	19° field		
−3.88		6.17	−0.79	5.41
−3.71		6.84	−0.48	6.46
−3.35		7.76	−0.08	7.31
−2.99	4.60	8.07	0.33	8.00
−2.61	6.49	8.69	0.72	8.79
−2.22	6.84	9.00	1.08	8.85
−1.85	7.55	—	1.44	9.06
−1.50	7.48	9.62	1.84	9.57
−0.77	8.51	10.1	2.25	9.84
−0.38	—	10.9	2.68	10.3
−0.03	10.6	13.5	3.04	11.7
0.31	11.7	15.2	3.39	13.5
0.67	13.7	16.4	3.78	15.6
1.03	15.4	19.8	4.18	16.7
1.80	17.1	21.0	4.59	18.5
2.52	18.8	20.8		
3.25	17.7	21.0		

thus function at the higher intensities since in order to produce a given amount of photoproduct, the intensity required varies inversely as the concentration of photosensitive material.

There is evidence in the studies with the normal eye that makes one believe in the existence of these high intensity rods even in the normal eye. Examination of Figs. 7 and 9 shows that with blue and violet light, there always are some points which fit neither on the cone nor the rod curves. These points do not appear when light of green or longer wavelengths is used. It is possible that these points represent the function of the high intensity rods because they come off the rod curve in the normal eye at about the place on the flat maximum
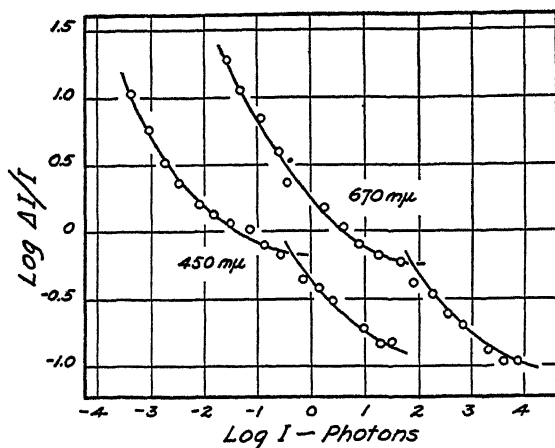
FIG. 8. Intensity discrimination for the colorblind eye. Two functions are present; these are the same with extreme red or violet light.
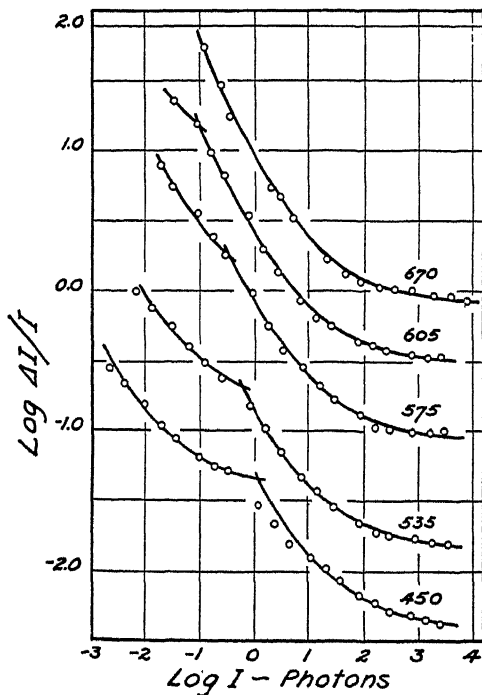
FIG. 9. Intensity discrimination for different spectral regions for the normal eye. Rod function is absent with red light but appears with the other colors. The ordinates apply to the data for yellow light in the middle; those for orange and red have been raised 0.5 and 1.0 log units respectively, and those for green and blue have been lowered 0.5 and 1.0 units respectively.

portion where they appear in the data of the colorblind. Obviously, the moment we use light in which the rod curve becomes less extensive so that these high intensity points fall beyond the rod-cone intersection point, they would no longer become evident in the normal data, and that indeed is the case.

SUMMARY

1. The visual functions of a completely colorblind individual are compared with those of the normal. The sensibility distribution in the spectrum has a maximum at 520 mμ at all brightnesses and thus corresponds to rod vision alone. This is confirmed by studies of dark adaptation which show final thresholds like those usually found for rod vision. Dark adaptation, measured both centrally and peripherally in the retina, is a single continuous function, and regardless of the brightness of the preceding light adaptation, is of the rapid type only, such as that found for the normal following low light adaptation. Visual acuity also shows a single continuous function like that for rod vision.

2. Both critical fusion frequency and intensity discrimination show two sections, one at low and the other at high intensities with a sharp transition from one to the other. Intensity discrimination is as good as for the normal eye, and covers much the same range. The maximal critical fusion frequency is only about 20 cycles per second as compared to 55 cycles for the normal.

3. The two sections shown by the colorblind eye for intensity discrimination and fusion frequency possess the spectral sensitivity of rod vision since the relative positions on the intensity scale are not influenced by using different parts of the spectrum.

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THE PROTECTIVE EFFECT OF SODIUM CHLORIDE AGAINST THERMAL DESTRUCTION OF THE PHAGE-FORMING MECHANISM IN STAPHYLOCOCCI

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It was shown several years ago that staphylococci which had been "activated" by rapid growth under favorable conditions and subsequently reduced to a resting state could produce an immediate six- to tenfold rise in [phage] when added to a phage-containing solution (1, 2). The basic experiment for demonstrating the phenomenon was performed by:

1. Growing the staphylococci in oxygenated broth at 36°C. for 2 hours.
2. Washing the cells and resuspending them in Locke's solution.
3. Maintaining the suspension at 5°C. for 2 hours.
4. Adding 4 ml. of the cell suspension to 1 ml. of phage diluted in Locke's solution to a titre of 1×10^9 activity units/ml.
5. Titrating various dilutions of the cell-phage mixture by the activity assay method (3).

The characteristic increase in [phage] occurred within 0.1 hour after adding the cells to the phage. This phage-augmenting property of staphylococci having a history of rapid growth in a favorable medium was not present in normal viable cells harvested 16 to 18 hours after inoculation of an agar substrate. As a working hypothesis it was postulated that the reaction between "activated" bacteria and phage involved some sort of a precursor which upon contact with phage was autocatalytically transformed into phage.

The capacity of "activated" organisms to increase [phage $_\text{activity}$] is abolished by concentrations of iodoacetate and methylene blue which leave the cells viable and reproductive. This is true also for exposure to heat and to sonic waves.

In the present paper we wish to report experiments in which the rapid destruction of phage "precursor" by heat was considerably retarded by the presence of sodium chloride.

Experimental Methods

The experiments consisted of exposing "activated" and normal bacteria suspended either in sterile distilled water or in 1 M NaCl solution to a temperature of 44°C. Following such thermal treatment the suspensions were chilled in an ice-water bath, and phage was then added to yield an initial concentration of 2×10^8 activity units per ml. These suspensions were again iced for an additional 30 minutes to permit inter-

action of "precursor" and added phage before preparing dilutions for activity assay. To determine the proportion of surviving bacteria following heat treatment, viable counts were performed at the beginning and at the end of the experiment, using suspensions which did not contain phage.

"Activated" staphylococci were prepared by growing an 18 hour culture of *Staphylococcus aureus* (the K strain) in a flask of tryptose phosphate broth. Continuous agitation of the medium at 36°C. was accomplished by means of a mechanical shaker mounted in a water bath. "Activation" was usually complete after 1½ hours of growth, at which time the suspension attained a concentration of approximately 1 × 10^9 bacteria per ml. Subsequent to "activation" the suspensions were centrifuged and the deposits resuspended in either sterile distilled water or in 1 M NaCl solution and adjusted to a density of 5 × 10^8 bacteria per ml. Normal "non-activated" bacterial suspensions were prepared in a similar manner except that the period of rapid growth was omitted. The "activated" and normal suspensions were then placed in a 44°C. water bath and 4 ml. samples were removed as soon as they had attained 44°C.; further 4 ml. samples were removed after 15 minutes at this temperature. As controls, 4 ml. samples were also taken prior to thermal treatment. All samples were chilled in ice-water bath before adding 1 ml. of phage (the K phage) containing 1 × 10^9 activity units per ml. Immediately after the addition of phage the samples were placed in an ice-water bath for 30 minutes; dilutions were then prepared for activity titration according to the method of Krueger. The total number of bacteria present in the preparations was determined by means of a Klett-Summerson photoelectric colorimeter which had previously been calibrated with cell suspensions of known density. Viable counts were determined by plating proper dilutions in tryptose agar; counts were made after incubation at 36°C. for 48 hours.

DISCUSSION OF EXPERIMENTAL RESULTS

Table I illustrates the results obtained in three individual experiments. Normal "non-activated" staphylococci which are suspended in water and exposed to a temperature of 44° C. for 15 minutes are no different from bacteria which are not subjected to thermal treatment. This is evidenced by the fact that there is no significant change in the amount of phage present when the cells are added to phage-containing solutions, as a test for "precursor," and by the fact that the number of surviving bacteria shows only a slight decrease.

In marked contrast is the behavior of "activated" bacteria suspended in water. After 15 minutes at 44° C. there is a very noticeable drop in the number of phage activity units, the loss ranging from 15- to 71-fold; concomitantly, there is a considerable reduction in the number of viable staphylococci, the approximate number of survivors being only 1 to 3.5 per cent of the original concentration. Microscopic examination of the bacteria suspended in sodium chloride solution shows no aggregation of the staphylococci, indicating that this is not the factor responsible for the drop in plate count.

Normal "non-activated" staphylococci, when suspended in 1 M NaCl solution, show practically no change either in phage formed or in the number of

viable bacteria remaining. However, "activated" staphylococci, under these conditions are able to maintain their state of activation without significant reduction in the number of surviving staphylococci.

Apparently 1 M NaCl effectively protects the "precursor" content of "activated" bacteria against thermal inactivation and also preserves the viability of the cells. Various electrolyte effects on the phage-bacterium reaction have been observed previously. Thus, Krueger and West (4) found that minute

TABLE I

Effect of Thermal Treatment on Suspensions of "Activated" and Normal Staphylococci in Distilled Water and in Sodium Chloride Solution

Suspension	Diluent	Exposure	Activity units/ml.			Viable bacteria/ml.		
			Experiment 1	Experiment 2	Experiment 3	Experiment 1	Experiment 2	Experiment 3
			$\times 10^8$	$\times 10^8$	$\times 10^8$	$\times 10^8$	$\times 10^8$	$\times 10^8$
		min.						
Non-activated	Water	Unheated	1.0	0.56	1.4	3.2	4.4	3.5
"	"	0	1.0	0.56	1.4	—	—	—
"	"	15	1.0	0.32	1.4	1.5	0.66	1.9
"Activated"	"	Unheated	7.0	32.0	14.0	2.9	2.2	2.4
"	"	0	7.0	32.0	14.0	—	—	—
"	"	15	0.48	0.45	0.56	0.10	0.02	0.02
Non-activated	NaCl	Unheated	1.0	0.56	1.4	—	—	—
"	"	0	1.0	0.56	1.4	—	—	—
"	"	15	1.0	0.17	1.4	3.2	5.6	3.3
"Activated"	"	Unheated	7.0	32.0	14.0	—	—	—
"	"	0	7.0	32.0	14.0	—	—	—
"	"	15	5.6	22.0	10.0	2.3	2.5	2.1

The symbol — signifies that the sample was not tested.
The initial phage concentration in all samples was 2.0×10^8 activity units/ml.

concentrations of Mn^{++} accelerated bacteriophagy by lowering the ratio of phage to bacteria requisite for massive lysis. Scribner and Krueger (5) observed that the addition of 0.25 M NaCl raised the lytic threshold five- to tenfold and thereby increased the end titre of the lysate. Krueger and Strietmann (6) noted similar results in studies on the phage-bacterium reaction conducted in the presence of Na_2SO_4.

SUMMARY AND CONCLUSIONS

Staphylococci which have been allowed to grow rapidly in a favorable environment and subsequently have been maintained in a resting state character-

istically produce a sharp rise in [phage] activity titre when added to phage. This capacity is quickly lost when the cells are suspended in distilled water and are exposed to 44° C. for a period of 15 minutes; at the same time the viable count drops to approximately 1 to 3.5 per cent of the initial value. 1 M NaCl protects "activated" cells against thermal destruction and preserves the phage-augmenting property. "Non-activated" staphylococci in distilled water suspension do not show this thermolability.

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OBSERVATIONS ON THE EFFECT OF PENICILLIN ON THE REACTION BETWEEN PHAGE AND STAPHYLOCOCCI

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In an earlier paper, Krueger, Cohn, and Noble (1) reported certain changes in the reaction between phage and *Staphylococcus aureus* brought about by the presence of penicillin. Particular attention was paid to the following points: (1) When phage acts on a broth suspension of staphylococci, the time required for initiation of massive lysis is considerably reduced by penicillin. (2) With values of $[phage]_0$ between 1×10^9 plaques/ml. and 1×10^7 plaques/ml. the accelerating action is independent of [penicillin] over a fairly wide range. (3) Exposure of the cellular substrate to penicillin action for at least 0.9 hour is essential to secure the full acceleration effect. (4) In some mixtures of phage, bacteria, and penicillin, phage formation apparently occurs in the absence of bacterial reproduction.

Because these experiments were performed with insensitive procedures for detecting increases in [bacteria] they were repeated later in modified form, and it was found that phage can be produced under conditions precluding multiplication of staphylococci.

We wish to present here an account of additional experimental work on the same general topic not reported in our first paper.

Experimental Methods

Throughout this paper the following abbreviations are used: P = the K race of staphylococcal phage; [P] = concentration of phage in plaques/ml.; B = the K strain of *Staphylococcus aureus;* [B] = bacteria/ml. Subscripts are employed as follows to indicate the procedure used for determining cell concentrations: $[B]_D$ = direct count; $[B]_V$ = viable count; $[B]_K$ = total cell concentration secured with Klett-Summerson photoelectric colorimeter; $[B]_C$ = direct visual comparison with formalinized cell suspensions of known density; PN = sodium penicillin G (crystalline); [PN] = concentration of sodium penicillin G in Oxford units.

1. Preparation of Cell Suspensions.—Stock cultures of staphylococci were grown in Roux flasks on tryptose agar for 18 hours at 36°C. To produce "activated" cell suspensions, tryptose-phosphate broth was inoculated with staphylococci recently harvested from Roux flasks and rapid growth was promoted by shaking the flasks in a water bath set at 36°C. When growth had progressed to a level of 1×10^9 bacteria/ ml. the suspensions were removed from the water bath and were used for the experiments described below.

2. Determination of Bacteria.—For many purposes, such as establishing the time of half-lysis, it was found that direct visual comparison with formalinized broth suspensions of staphylococci was adequate ([B]$_C$). Actually, very good agreement was obtained between values secured by direct visual comparison and by the Klett-Summerson colorimeter.

Direct counts in the Petroff-Hausser chamber were routinely made when [B] was >5 × 10^7 staphylococci/ml. In the presence of phage, the cell suspensions were diluted in formalinized broth to inhibit continuing lysis.

In using the Klett-Summerson photoelectric colorimeter for determining [B], readings were converted into cells/ml. by reference to a standard curve plotted from points established by direct counts.

Viable counts were made by plating aliquots of test suspensions on the surface of tryptose agar plates taking care to spread the fluid evenly. On occasion, pour plates were used but these required longer incubation for reading and appeared to possess little advantage.

Determinations of phage were carried out by Gratia's method (2), using a separate pipette for each serial dilution.

EXPERIMENTAL RESULTS

1. Quantitative Aspects of the Penicillin Effect.—In order to ascertain the effects of varying [PN] and [P] on the time of lysis of mixtures containing bacteria, phage, and penicillin, several series of experiments were carried out using the standard "activated" cell suspension in tryptose-phosphate broth as a substrate. Ten ml. test mixtures were prepared, consisting of 5 ml. of cell suspension (1 × 10^9 bacteria/ml.), 1 ml. of phage solution, 1 ml. of penicillin solution, and 3 ml. of broth. These were placed in a 36° water bath and were shaken mechanically to maintain even suspensions. Turbidity readings were taken every 0.2 hour by direct visual comparison and the elapsed times required to reduce [B] to one-half the original value were recorded. The results are summarized in Table I.

The minimal [PN] producing an effect on time of lysis appears to be 1 × 10^{-2} units/ml. This amount in the absence of phage does not produce measurable lysis within the arbitrary period of 5 hours established as the limit of observation. Combined with [P]'s of 1 × 10^5 plaques/ml. or 1 × 10^6 plaques/ml., the times of half-lysis are respectively 5.1 and 4 hours; controls without PN and with [P] = 1 × 10^5 plaques/ml. do not lyse. With [P] = 1 × 10^6 plaques/ml. half-lysis occurs between 4.0 and 5.0 hours.

When higher concentrations of phage are employed; namely, 1 × 10^7 or 1 × 10^8 plaques/ml. the controls without PN lyse at 2.3–3.8 hours and 1.6 hours respectively while the suspensions containing both P and PN show moderate acceleration.

With the highest [P] tested, namely 1 × 10^9 plaques/ml., lysis proceeds at the same rate in control and test suspensions.

As [PN] is raised above 0.1 unit/ml. the lower concentrations of P (1×10^4, 1×10^5, and 1×10^6 plaques/ml.) appear to have little effect in accelerating lysis beyond the rates for PN alone.   However, with [P] = 1×10^7 there is a pronounced speeding of lysis in the test mixtures as compared with PN alone or P alone.

Nearly all the suspensions made with a [P] of 1×10^8 plaques/ml. attain half-lysis by 0.8 hour.   This is significantly less than the t (half-lysis) for 1×10^8 phage alone (1.6 hours) and as much as 1.9 hours less than the t (half-lysis) observed for 1×10^3 units PN alone (2.7 hours).   When [P] = 1×10^9 plaques/ml.

TABLE I

Time of Half-Lysis in Mixtures of Phage, Penicillin, and Staphylococci in Tryptose Phosphate Broth at 36°C.

[Bacteria] determined by direct comparison with standards containing known concentrations of staphylococci.

[Penicillin] units/ml.	[Phage], *plaques/ml.*						
	0	1×10^4	1×10^5	1×10^6	1×10^7	1×10^8	1×10^9
	hrs.	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*
0		5.0	5.0	From 4.0 to 5.0 hrs.	From 2.3 to 3.8 hrs.	1.6	0.8
1×10^{-2}	Usually no lysis to 5.0 hrs.	Usually no lysis to 5.0 hrs.	5.1	4.0	2.1	1.2	0.8
1×10^{-1}	1.9	1.7	1.7	1.5	1.2	0.8	0.8
1.0	2.1	2.0	1.7	1.6	1.3	0.8	0.7
10.0	2.1	2.1	2.0	1.9	1.3	0.8	0.7
1×10^2	2.5	2.5	2.3	2.0	1.3	0.8	0.7
1×10^3	2.7	2.7	2.4	2.2	1.3	0.8	0.7
1×10^4	2.7	2.5	2.5	2.3	1.8	0.9	0.7

t (half-lysis) is only 0.7 hour in the five mixtures having the highest [PN]'s. Evidently, the presence of PN exerts little influence on the rate of lysis in suspensions containing so much P, for the P control reaches half-lysis in 0.8 hour.

A somewhat broader view of the accelerative effect of penicillin can be obtained by comparing Fig. 1 with Fig. 2.   Fig. 1 shows the lytic action on the standard cell suspension of [P]'s varying from 1×10^9 to 1×10^6 plaques/ml.; the curves for lysis are based on photoelectric colorimeter measurements secured in four separate experiments.   Fig. 2 presents the data from a single experiment in which [PN] = 1×10^2 units/ml. and [P] varies from 1×10^9 to 1×10^4 plaques/ml.   It is evident that the lytic process proceeds more rapidly when PN is present than when P acts alone.

PN alone lyses the test organism.   In mixtures containing [PN]'s ranging
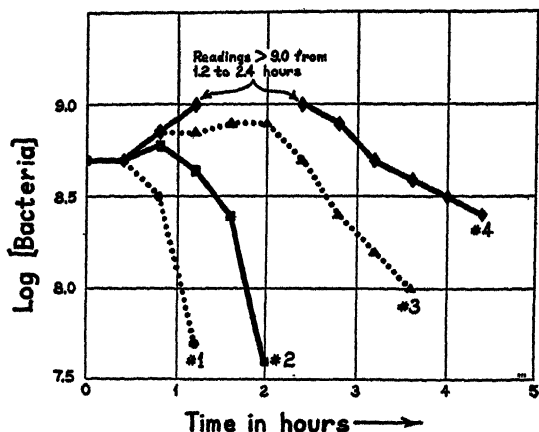
FIG. 1. Lytic curves for staphylococci and phage in tryptose-phosphate broth at 36°C. without penicillin. Points are averages of four experiments. Initial [bacteria]: 5×10^8/ml. Initial concentrations of phage: No. 1 = 1×10^9; No. 2 = 1×10^8; No. 3 = 1×10^7; No. 4 = 1×10^6. [Bacteria] determined by Klett-Summerson photoelectric colorimeter. Final [phage] after lysis (plaques/ml.): No. 1 = 5×10^9; No. 2 = 5×10^9; No. 3 = 4×10^9; No. 4 = 2×10^8.
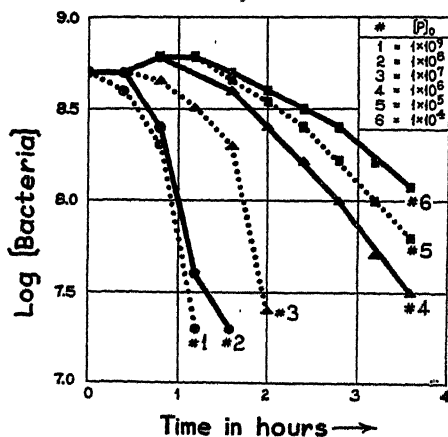
FIG. 2. Lytic curves for staphylococci + phage + penicillin in tryptose-phosphate broth at 36°C. Data of a single experiment. Initial [bacteria]: 5×10^8/ml. Initial [penicillin]: 1×10^2 units/ml. Initial concentrations of phage varied from 1×10^9 plaques/ml. to 1×10^4 plaques/ml. [Bacteria] determined by Klett-Summerson photoelectric colorimeter.

from 0.1 unit/ml. to 1×10^4 units/ml., the t (half-lysis) varies inversely with $[PN]_0$. This parallels Eagle's observations that bacteria were killed more rapidly with lower [PN]'s than with relatively concentrated PN (3).

2. The Production of Phage in Broth Mixtures of Phage, Staphylococci, and Penicillin.—In order to follow the course of P formation, plaque counts were made at brief intervals on samples taken from mixtures of P, PN, and B in tryptose-phosphate broth at 36°C. Four experiments were done employing

Time in hours⟶

FIG. 3.

Time in hours⟶
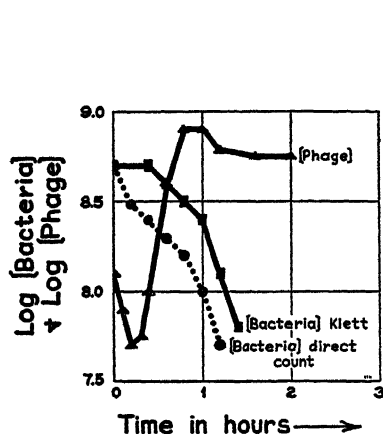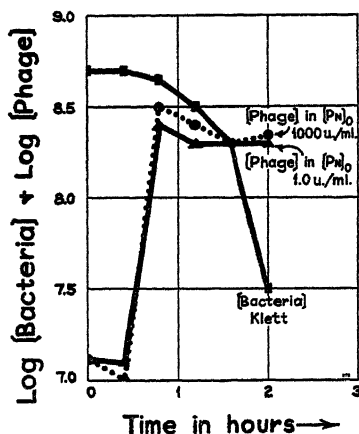
FIG. 4.

FIG. 3. Curves for cellular lysis and phage formation in mixtures of staphylococci, phage, and penicillin in tryptose-phosphate broth at 36°C. (average of four experiments). "Activated" bacteria kept 1 hour at 21°C. and 0.5 hour at 5°C. Centrifuged and sediment suspended in broth containing 1×10^4 units PN/ml. and about 1×10^8 plaques/ml. Mixture shaken at 36°C. and samples taken at intervals for: (1) determination of [bacteria] by photoelectric colorimeter and by direct count, and (2) phage assay. Latter aliquots immediately diluted at 5°C.

FIG. 4. Curves for cellular lysis and phage formation in mixture of staphylococci, phage, and penicillin in tryptose-phosphate broth at 36°C. "Activated" bacteria kept 1 hour at 21°C. and 0.5 hour at 5°C. Centrifuged and sediment suspended in broth containing (a) 1.3×10^7 plaques/ml. and 1 unit PN/ml. and (b) 1.3×10^7 plaques/ml. and 1,000 units PN/ml. Samples taken at intervals for (1) determination of [bacteria] by photoelectric colorimeter, and (2) phage assay. Lytic curves for [PN]$_0$ 1.0 and 1×10^3 identical. Phage curves almost identical.

the concentrations shown in Fig. 3. The curve for [P] drops sharply to 0.2 hour and then rises rapidly to a peak at 0.8 hour. The maximal [P] is approximately six times the [P] initially present and sixteen times the lowest value reached during the reaction (at 0.2 hour). Subsequently, there is a 30 per cent decrease in [P] until a steady state is reached at 1.6 hours.

Judging from the [B]$_D$ curve, bacterial lysis begins as soon as the reactants are mixed and not only continues during the phase of P formation, but extends into the period when [P] falls off 30 per cent. The curve for [B]$_K$ (obtained by

means of the photoelectric colorimeter) shows a slower drop than that based on [B]$_D$; a possible reason for this discrepancy will be discussed later.

Other, essentially identical, experiments were performed with smaller concentrations of P and PN; the curves for B lysis so obtained agreed well with those presented in Fig. 2.   Fig. 4 illustrates such an experiment, in which two different mixtures were followed.   In each, [B]$_0$ was 5 $\times$ 10^8/ml. and [P]$_0$ 1.3 $\times$ 10^7 plaques/ml.; [PN]$_0$ in one was 1 unit/ml. and in the other 1 $\times$ 10^3 units/ml.   The rate of lysis is seen to be the same for both suspensions and there is no significant difference in the curves for P formation.   However, when the latter are compared with P curves secured with higher initial values of [P] (Fig. 3), certain differences are noticeable: (1) The maximal [P] formed is twenty-five times the initial [P] instead of six times this concentration.   (2) There is no drop in [P] during the early phases of the reaction.   (3) The period of P production is completed before massive cellular lysis is well under way.

In the experiments already described, P formation has occurred without demonstrable cellular reproduction, confirming earlier experiments performed with the K strain of *Staphylococcus aureus* in this laboratory and with *Staphylococcus muscae* in Dr. Winston Price's laboratory (4).   However, the suspending medium for the reaction between PN and B was veal infusion broth in Dr. Price's experiments and tryptose-phosphate broth in our experiments, each medium favoring the growth of the respective organism in the absence of PN. While it is known that in some cases at least (*Escherichia coli* and T2 phage) B stop multiplying as soon as they are infected with P (5) one must consider the possibility that a certain amount of cell division, perhaps restricted to P-infected cells, could occur without detection by the procedures employed for determining [B].   For example, in the experiment of Fig. 4 only 1 cell in 40 is infected at 0.4 hour when [P] begins to rise, so that a generalized single fission among P-containing staphylococci would result in a mere 2.5 per cent increase in [B].

One way to test the possibility that selective cell division may be involved is to raise the P/B ratio in order to infect a considerable percentage of the total bacteria present; then, if infected cells are prone to divide, the increase should be experimentally detectable.   This was done in the experiments summarized in Figs. 3, 5, and 6, unfortunately without settling the issue.   Apparently, as the P/B ratio is raised the early drop in [P] is exaggerated (Fig. 5) and may continue to practical extinction of the P added (Fig. 6).   In some cases there is measurable production of P but the final titre never exceeds the initial [P] (Fig. 5).

While the chance of any significant cell division seemed remote in the above experiments, we felt it would be advisable to limit this factor still further by attempting to produce P in mixtures of B, PN, and P suspended in Locke's solution.   These experiments are described below.

3. The Formation of Phage in Locke's Solution Mixtures of Phage, Staphylococci, and Penicillin Containing 5 to 10 Per Cent Broth.—The course of P production in Locke's solution mixtures of P, B, and PN was obser ved as follows:

"Activated" B were centrifuged down from the tryptose-phosphate medium and were resuspended at a concentration of 4×10^8 cells/ml. in Locke's solution con-
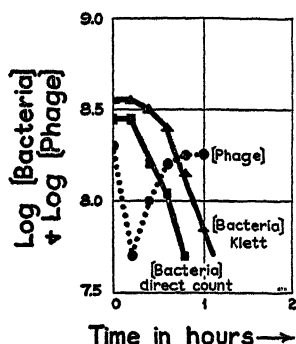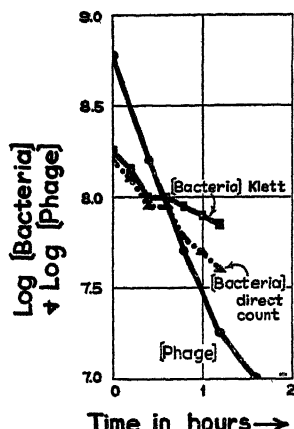
Fig. 5   Fig. 6

FIG. 5. Curves for cellular lysis and phage formation in mixture of staphylococci, phage, and penicillin in tryptose-phosphate broth at 36°C. "Activated" bacteria kept 1 hour at 21°C. and 0.5 hour at 5°C. Centrifuged and sediment suspended in broth containing 2×10^8 plaques/ml., 1×10^4 units PN/ml., and 7×10^8 B/ml. Mixture shaken at 36°C. and samples taken at intervals for: (1) determination of [bacteria] by photoelectric colorimeter and by direct count, and (2) phage assay.

FIG. 6. Curves for cellular lysis and phage formation in mixture of staphylococci, phage, and penicillin in tryptose-phosphate broth at 36°C. "Activated" bacteria kept 1 hour at 21°C. and 0.5 hour at 5°C. Centrifuged and sediment suspended in borth containing 7×10^8 plaques/ml., 1×10^4 units PN/ml., and 2×10^8 B/ml. Mixture shaken at 36°C. and samples taken at intervals for: (1) determination of [bacteria] by photoelectric colorimeter and by direct count, and (2) phage assay.

taining 1.0 unit PN/ml. The suspension was held at 5°C. for 1 hour. At this time P was added (usually <1 ml. to each 9 ml. of the Locke's solution mixture) and the preparation was kept an additional hour at 5°C. It was then divided into 10 ml. aliquots in test tubes and the latter were shaken at 36°C. Samples were removed at intervals for plaque counts and for determination of $[B]_K$. The phage samples were diluted immediately in broth or Locke's solution at 5°C., the samples for $[B]_K$ were diluted in Locke's solution at 5°C. and were read at once.

Fig. 7 is a summarized graphic presentation of the data from four experiments. Cellular lysis without an increase in the total cell count begins shortly

after the mixtures containing PN are brought to 36°C. In the absence of PN, however, considerable B growth takes place and lysis is much slower.

The [P] curve shows a marked drop during the 1 hour period at 5°C. and rises sharply when the temperature is adjusted to 36°C. Some 60 per cent of the total P produced is formed during the phase of massive bacterial lysis (taking place between 2.6 hours and 3.4 hours). The yield of P is not remarkable, being only 6 × [P]$_0$.
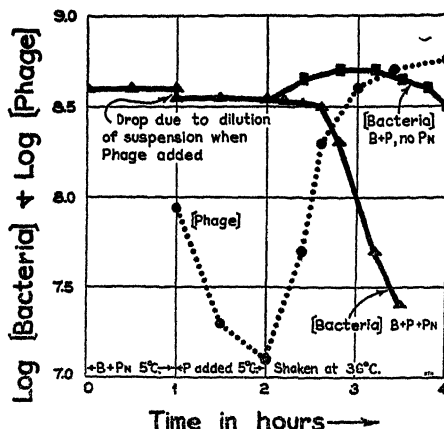
FIG. 7. Curves for phage formation and cellular lysis in Locke's solution. "Activated" bacteria 4 × 10^8/ml. held at 5°C. 1 hour in Locke's solution containing 1.0 unit PN/ml. Phage diluted in Locke's solution added and mixture kept additional hour at 5°C. Suspension transferred to tubes and shaken at 36°C. Samples taken at intervals for plaque counts and determination of [bacteria] by photoelectric colorimeter. Aliquots for phage assay immediately diluted at 5°C. The curves for [bacteria], B + P + PN, and [phage] are averages of four experiments; that for [bacteria], B + P, is an average of two experiments.

To confirm the lack of cellular reproduction noted above, an additional set of experiments was performed using parallel determinations of]B] by the photoelectric colorimeter and by direct count. The data are presented in Fig. 8. Again, as noted earlier in connection with the broth mixtures, there is a noticeable inclination of [B]$_D$ to drop in advance of [B]$_K$; both curves exhibit no evidence of an increase in [B]. Plaque counts were done only at the start and at the end of the experiments; they showed an average increase of four times [P]$_0$.

While it is reasonable to expect that cell multiplication would be discouraged in the experiments employing Locke's solution as a suspending medium, the inclusion of some nutrients cannot be overlooked. Concentrations of tryptose-phosphate broth between 5 and 10 per cent were added with the P and it is clear from the control curve (no PN) for [B] in Fig. 7 that even this dilute

medium suffices to support staphylococcal growth, providing no PN is present. However, when the test mixture contains both P and PN, the curve for [B] determined by direct counts shows a prompt and continuing downward trend.

Price has performed similar experiments with *S. muscae* (6), using suspensions of B, PN, and P in broth diluted 1/17 with Locke's solution. Since P formation was his primary concern no B growth curves are given and comparison with our data cannot be made.
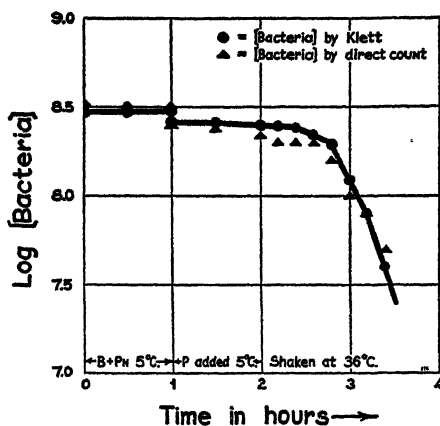
FIG. 8. Lysis without cellular growth in mixture of phage, penicillin, and staphylococci in Locke's solution. "Activated" bacteria 3.2×10^8/ml. held at 5°C. 1 hour in Locke's solution containing 1.0 unit PN/ml. Phage diluted in Locke's solution added to produce $[P]_0$ of 1×10^8 plaques/ml. and mixture kept additional hour at 5°C. Suspension transferred to tubes and shaken at 36°C. Samples taken at intervals for determination of [bacteria] by Klett-Summerson photoelectric colorimeter and by direct count (formalinized Locke's solution used as diluent for latter). Curve drawn through averaged values for three sets of Klett determinations. Direct count points are averages of these same three experiments and an additional one for which Klett values were not determined.

4. Mutual Sensitization of Staphylococci by Phage and Penicillin.—

(*a*) *Preliminary Treatment of Cells with Penicillin Followed by Exposure to Phage:* Several experiments were performed to determine whether a period of exposure to PN made staphylococci susceptible to accelerated lysis when removed and suspended in P. Table II summarizes one of these tests, in which the initial mixture consisted of 5×10^8 B/ml. and 10 units PN/ml. The suspension was kept at 5°C.; at intervals two 10 ml. aliquots were removed and centrifuged for 10 minutes at 5°C. One sediment was resuspended in broth; the other in P containing 1×10^7 plaques/ml. and both were shaken at 36°C. $[B]_D$ was determined frequently to establish the course of lysis, and the time of half-lysis was recorded for each mixture.

It appears that the cells exposed to 10 units PN/ml. at 5°C. become sensitive

to the action of P and that this sensitization process requires approximately 1 hour to produce maximal acceleration of lysis.

This type of experiment is open to the theoretical objection that small amounts of PN may be carried over into the final mixtures and from the evidence presented in Table I it is known that concentrations between 1×10^{-1} and 1×10^{-2} unit PN/ml. could speed up the lytic process. Probably such a mechanism does not apply to the present case for no lysis of the cells resuspended in broth occurs within the 6 hour period of observation.

TABLE II

Sensitization of Staphylococci to Phage Action by Previous Exposure to Penicillin

Preliminary Mixtures: $[B]_0$ 5×10^8; $[PN]_0$ 10 units/ml. Kept at 5°C. At various intervals two samples removed, centrifuged in angle centrifuge at 4,000 R.P.M. (at 5°C.). Supernatants decanted and sediments resuspended: (*a*) in broth, (*b*) in P of 1×10^7 plaques/ml. Course of lysis followed by comparison with B suspensions of known density.

Resuspended Mixtures: (In shaker at 36°C.)

Period of exposure to PN (including time of centrifugation)	Time of half-lysis (from time resuspended)	
	Broth suspension	Phage suspension
min.		
10	No lysis to 6.0 hrs.	3.4 hrs.
34	" " " 6.0 "	2.5 "
58	" " " 6.0 "	1.9 "
82	" " " 6.0 "	2.0 "

Controls: (Shaken at 36°C.)

$[B]_0$	$[PN]_0$	$[P]_0$	Time of half-lysis
			hrs.
5×10^8	0	1×10^7	3.7
5×10^8	10 units/ml.	0	2.2
5×10^8	10 " "	1×10^7	1.7

(*b*) *Preliminary Treatment of Cells with Phage Followed by Exposure to Penicillin:* It is not possible to determine experimentally whether P treatment conditions stapyylococci to subsequent action by PN. The difficulty encountered is that during preliminary exposure of the cells they rapidly adsorb P and carry it over into the second phase of the experiment when the B are resuspended in PN solution. Consequently, in effect, one is simply making mixtures of bacteria, phage, and penicillin.

5. Experiment to Detect Cellular Swelling during Reaction between Phage, Staphylococci, and Penicillin.—During the reaction between P, B, and PN we regularly observed a considerable lag in the curve for cellular lysis plotted from $[B]_K$ data when compared with that based on $[B]_D$ data (Figs. 3, 5, and 6). It seemed conceivable that this discrepancy might depend upon swelling of the cellular substrate prior to lysis, with the result that the turbidity readings

would remain high while the direct count values would drop. To test this idea the following experiment was performed:

"Activated" staphylococci were prepared in the usual fashion and were held at 5°C. for 1 hour. A mixture in tryptose-phosphate broth was made containing: 5×10^8 B/ml., 1×10^8 P plaques/ml., and 1×10^3 units PN/ml. It was shaken at 36°C. and at intervals samples were removed for: (1) determination of $[B]_K$; (2) determination of $[B]_D$; (3) microscopic measurement of cell size (wet preparations) with the ocular micrometer.
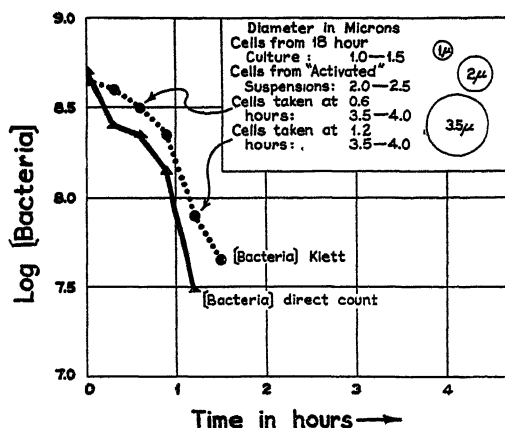
FIG. 9. Swelling of staphylococci during reaction with phage and penicillin. $[B]_0 = 5 \times 10^8$/ml. $[P]_0 = 1 \times 10^8$ plaques/ml. $[PN]_0 = 1 \times 10^3$ units/ml. Tryptose-phosphate broth at 36°C. Samples removed at intervals for determination of $[B]_D$ and $[B]_K$ and for microscopic measurement of cells with ocular·micrometer.

The curves for $[B]_D$ and $[B]_K$ are plotted in Fig. 9 and the diameters of the staphylococci are recorded. Measureable swelling of the organisms occurs during the activation procedure (from an average diameter of 1.25 microns to one of 2.25 microns). A further increase to an average diameter of 3.75 develops by 0.6 hour from the time of mixing B, P, and PN. This appears to be the maximal amount of swelling, for samples taken at 1.2 hours give identical measurements.

SUMMARY AND CONCLUSIONS

The essential facts relating to the reaction between phage, sodium penicillin G, and the K race of *Staphylococcus aureus* are:

1. Except when [P] is very high, massive lysis of the cellular substrate occurs considerably sooner in the P-PN-B mixture than in preparations containing P alone or PN alone.

2. The accelerative effect is present in concentrations of PN varying from 0.1 to 1×10^4 units/ml.

3. Acceleration of lysis can be secured by exposing staphylococci to PN prior to treatment with P.

4. In certain concentrations of P and B in tryptose-phosphate broth, P formation apparently takes place without bacterial reproduction. The extent to which P is produced is influenced very little by $[PN]_0$ but is markedly dependent upon $[P]_0$. With low P/B ratios the $[P]$ curve shows a lag followed by a rapid rise to a peak of 25 to 30 times $[P]_0$. When the P/B ratio approaches unity there is a considerable primary drop in $[P]$ and later an increase which, however, fails to bring the total P produced above $[P]_0$. When P/B is still higher, the $[P]$ curve drops profoundly as the bacteria lyse and never enters into a productive phase.

5. In Locke's solution mixtures of P-PN-B, containing 5 to 10 per cent broth, P formation occurs in the absence of detectable cellular reproduction to the extent of a four- to sixfold increase over $[P]_0$.

6. Direct microscopic examination of wet preparations removed during the P-PN-B reaction has disclosed swelling of the staphylococci. The swollen cells are three times the diameter of normal *S. aureus* secured from an 18 hour culture. Cellular swelling apparently accounts for the experimental observation that the curve for lysis plotted from $[B]_K$ lags considerably behind the $[B]_D$ curve. Increase in the size of individual cells would tend to keep the photoelectric colorimeter measurements high even while the direct count was diminishing.

7. When the P-PN-B reaction is carried out in broth, attainment of the peak in P production is followed by a moderate loss of P. This does not occur when P, PN, and B react in Locke's solution.

The reaction dealt with here between P, PN, and *S. aureus* is similar in a good many respects to that investigated by Price for P, PN, and *S. muscae*. For example, in both cases P is produced without bacterial reproduction. There are, however, certain noteworthy differences: (*a*) PN increases the time of half-lysis for *S. muscae* and lessens it for *S. aureus*. (*b*) The yields of P in ranges of $[P]$ and $[PN]$ permitting P formation without bacterial reproduction are higher for *S. muscae* than for *S. aureus*. (*c*) An increase in $[PN]$ from 33 units/ml. to 833 units/ml. greatly reduces the final plaque count secured with' *S. muscae* as a substrate but has no discernible influence on the reaction when *S. aureus* is used.

Currently studies are in progress on the P-PN-B reaction in a synthetic medium in order to obtain information on the mechanism involved.

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THE SYNTHESIS FROM VITAMIN A₁ OF "RETINENE₁" AND OF A NEW 545 mμ CHROMOGEN YIELDING LIGHT-SENSITIVE PRODUCTS*

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I

INTRODUCTION

The bleaching of rhodopsin in the retina liberates the yellow carotenoid retinene₁, which is subsequently converted to vitamin A₁ (Wald, 1935–36 a, b). Retinene₁ is characterized by an absorption maximum in chloroform solution at 387 mμ (365 mμ in hexane); and yields when mixed with antimony chloride a deep blue product possessing an absorption maximum in the red, at 664 mμ.

Recently R. A. Morton and his coworkers at the University of Liverpool have converted vitamin A₁ synthetically into a product which is comparable in absorp-
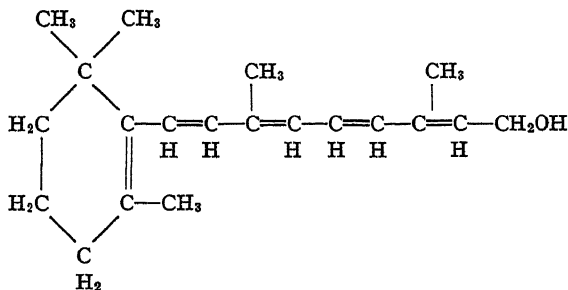
Vitamin A₁, $C_{20}H_{29}OH$
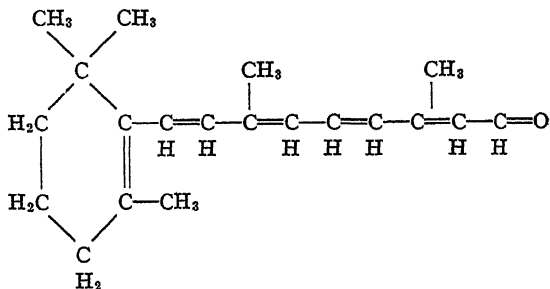
Vitamin A₁-aldehyde, $C_{20}H_{28}O$

tion spectrum and antimony chloride reaction with retinene$_1$ (Ball, Goodwin, and Morton, 1946). They believe this to be vitamin A_1-aldehyde. Morton (1944) had suggested this structure for retinene$_1$ earlier on theoretical grounds.

The presence of an aldehyde group in the synthetic product is substantiated by the preparation of a crystalline 2,4-dinitrophenylhydrazone, and by the formation of a silver mirror with Tollens's reagent (ammoniacal silver nitrate containing a trace of alkali). It is not yet certain, however, that this is the only departure from the structure of vitamin A_1; nor do the properties of Morton's product conform wholly with those reported for vitamin A_1-aldehyde synthesized by other means (Hunter and Hawkins, 1944; Van Dorp and Arens, 1947). The relations between the synthetic substance and retinene preparations from the retina are also not yet entirely clear.

Nevertheless Morton's contribution represents a signal advance. One can be confident that the synthetic product is closely related to if not identical with retinene$_1$. I shall refer to it below as "retinene$_1$," allowing the quotation marks to express such reservations as remain concerning its identity with the natural product.

In the simple procedure devised by Ball, Goodwin, and Morton, a petroleum ether solution of vitamin A_1 is let stand in the cold over solid manganese dioxide for 3 to 4 days, in the course of which most of it is transformed into "retinene$_1$."

On repeating this experiment I found that a basic element in the process is the powerful adsorption of vitamin A_1 by manganese dioxide. Unless the vitamin is present in excess, all of it is adsorbed out of solution; and no "retinene$_1$" ever takes its place. If after a time one elutes the manganese dioxide with ethanol in petroleum ether, one finds not "retinene$_1$" but a new substance which yields with antimony chloride a wine-colored product, with an absorption maximum at 545 mμ. I shall refer to this hereafter ás the 545 mμ chromogen.

The further study of these reactions led to a new procedure, by which "retinene$_1$" or the 545 mμ chromogen can be synthesized at will from vitamin A_1 in a matter of minutes. This may be characterized as a controlled *chromatographic oxidation*. In the present paper this process is described, spectral properties of natural retinene$_1$ are compared with those of the synthetic product, and a first account is given of the 545 mμ chromogen. From the latter substance colored products are obtained which strikingly resemble in some of their properties the visual photopigment rhodopsin, itself a natural derivative of vitamin A_1.

II

The Reaction

On examining the original procedure of Ball *et al.*, in which a petroleum ether solution of vitamin A_1 is exposed to solid manganese dioxide, I found the course

of the reaction to be as follows: Manganese dioxide adsorbs vitamin A_1 very strongly, transforming it rapidly to "retinene$_1$." The latter is much less strongly adsorbed, and so is displaced from the manganese dioxide surface by new vitamin A_1 as rapidly as it is formed. Hence one observes that it replaces vitamin A_1 in the supernatant solution. This happens, however, only in the presence of excess vitamin A_1. If no excess is present, the "retinene$_1$" remains adsorbed and is further transformed to the 545 mμ chromogen. Even when excess vitamin A_1 has been employed, and "retinene$_1$" appears in the supernatant, the final charge of vitamin A_1 on the manganese dioxide surface is retained to form the 545 mμ chromogen; and elution of the manganese dioxide at the end of the process yields this substance alone.

Having recognized this situation, I found that the entire procedure can be recast in chromatographic form. The manganese dioxide is packed in the familiar type of chromatographic column. A solution of vitamin A_1 in petroleum ether is poured in at the top, and the desired product is drawn off in the filtrate. Whether this is "retinene$_1$" or the 545 mμ chromogen depends only on whether, with a given amount of vitamin A_1, one uses a short or a long column.

For example, if one begins with a solution of 10 mg. of crystalline vitamin A_1 alcohol in petroleum ether, and about 0.6 gm. of manganese dioxide packed in a column about 1 cm. long and 1 cm. wide, one can pour the vitamin A_1 solution in at the top, wash with further petroleum ether, and within a few minutes draw off under light suction a concentrated solution of "retinene$_1$" as filtrate.

Alternatively, if one uses a longer column (*ca.* 2.5 gm. manganese dioxide in a column about 4 cm. long and 1 cm. wide) the vitamin A_1 is entirely adsorbed, and no carotenoid appears in the filtrate even on prolonged washing with petroleum ether. After washing for 20 to 30 minutes, elution of the column with 5 per cent ethanol in petroleum ether yields a bright yellow solution of the 545 mμ chromogen.

The sequence of the reactions can be demonstrated very simply. One can prepare "retinene$_1$" on a short column of manganese dioxide, then readsorb this on a second column of manganese dioxide. On elution of the latter one obtains the 545 mμ chromogen alone.

Under the circumstances described, the separation of products is virtually complete. The "retinene$_1$" contains little or no admixture of 545 mμ chromogen, the 545 mμ chromogen no observable "retinene$_1$." On elution of the 545 mμ chromogen one does obtain relatively small quantities of other substances, all of which possess absorption bands in chloroform maximal at 360 to 370 mμ, and all of which yield with antimony chloride purple or violet products with absorption bands maximal at 552 to 560 mμ. These are being investigated further.

<center>III</center>

<center>*"Retinene₁"*</center>

The proposal that natural retinene₁ is vitamin A₁-aldehyde rests at present primarily on its similarity with the synthetic product in spectrum and antimony chloride reaction. In deference to the observations of Morton's group on the latter substance, still largely unpublished, I should like to do no more in the present section than to confirm the extraordinary correspondences in spectrum between the natural and synthetic substances; and to raise one problem which involves a possible lack of complete identity between them.

Absorption spectra of preparations of the synthetic product and of retinene₁ from cattle retinas, both dissolved in chloroform, are shown in Fig. 1. The synthetic material had been partly purified by chromatographic adsorption on calcium carbonate, the cattle retinene₁ by adsorption on magnesium oxide. Both preparations have virtually identical spectra, with absorption maxima at about 387 mμ.

The retinenes exhibit peculiar displacements of spectrum with change of solvent, the structural implications of which are discussed below. The natural and synthetic substances maintain a close correspondence of spectrum in all the solvents which I have tried. In both types of preparation, the absorption maxima lie at about 380 mμ in absolute ethanol and at about 365 mμ in hexane.

The spectrum of the blue product obtained by mixing retinene₁ with antimony chloride reagent is also shown in Fig. 1. Practically identical spectra are obtained from the synthetic product and natural retinene₁, in this case from the squid retina. In both instances the absorption is maximal at 664 to 666 mμ, with a broad inflection in the region 610 to 615 mμ.

In the antimony chloride test with natural retinene₁, the density of color rises slowly to a maximum about 90 seconds after mixing the reagents (room temperatures), then slowly falls. The synthetic product displays the same type of behavior, at least to a first approximation.

In preparations of synthetic "retinene₁," the ratio of the extinction of the antimony chloride product at about 660 mμ to the extinction of the chloroform solution at 387 mμ, for equivalent concentrations of "retinene₁" measured in the same depth of layer, was 1.65–1.68. In a preparation of natural retinene₁ from cattle retinas the corresponding ratio was 1.69. (In these experiments the antimony chloride extinction was measured in the Pulfrich photometer (Zeiss), using the S66.6 filter. A measurement of the extinction at 664 mμ made in monochromatic light would have yielded a somewhat higher value, and hence also higher values of the above ratios—actually about 2.9.)

These correspondences are so close that one is tempted to conclude without more hesitation that the synthetic substance is identical with natural retinene₁. There are, however, several problems which remain to be clarified, among which I should like to mention one.
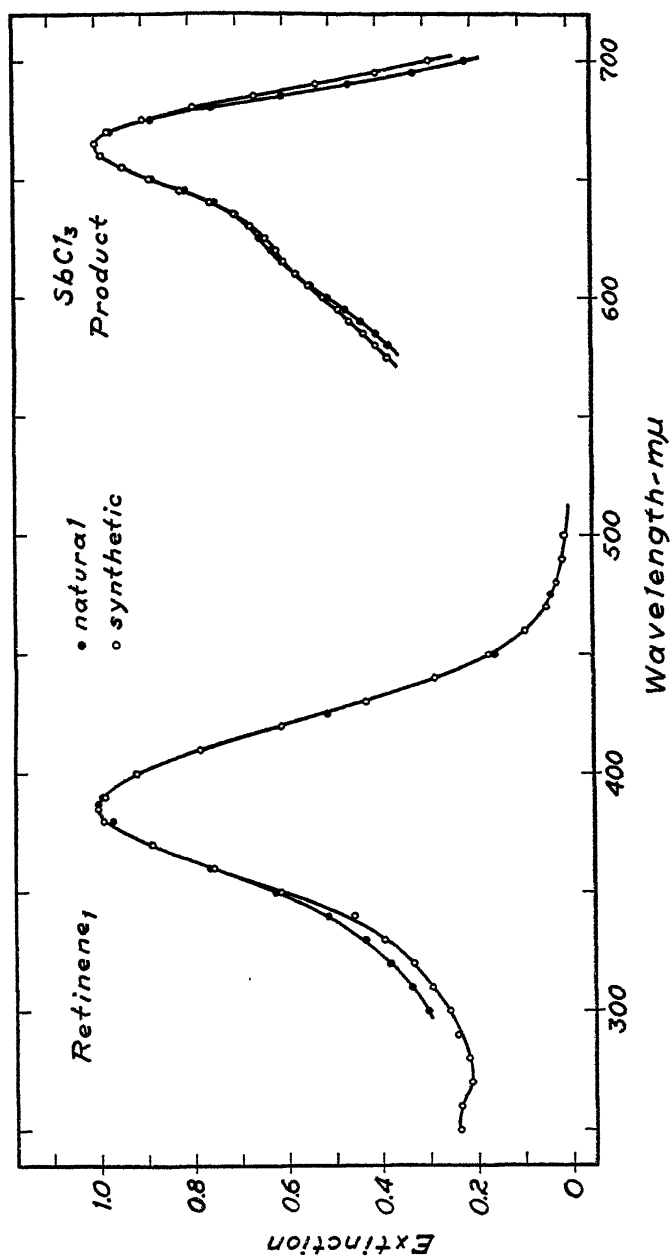
FIG. 1. Comparison of natural retinene₁ with the synthetic product. Absorption spectra of cattle retinene₁ in chloroform, and of the blue antimony chloride product of squid retinene, compared with similar preparations of the synthetic substance. The absorption is plotted as extinction or optical density, $\log I_o/I$, in which I_o is the incident and I the transmitted intensity.

Some time ago I found that natural preparations of retinene₁ behave as pH indicators, going from relative colorlessness in alkaline solution to deep yellow in acid, with a corresponding shift in absorption spectrum toward longer wavelengths in acid solution (Wald, 1937–38, pages 810–811). This is the primary basis of similar changes in the product of bleaching rhodopsin in solution (Chase, 1935–36); a product which has for this reason been called "indicator yellow" (Lythgoe, 1937), and which I believe to be a retinene₁-protein (Wald, 1937–38, page 813). Comparable changes in color with pH are observed in whole retinas (Wald, 1936–37, pages 50–51).

Unpurified preparations of natural retinene₁ dissolved in 1 per cent aqueous digitonin shift markedly in absorption spectrum with pH. The absorption maximum lies at about 380 mμ at pH 7, about 385 mμ at pH 4, and about 366 mμ at pH 9.4. The maximum extinction is also highest in alkaline solution, the spectrum growing lower and broader as the solution is acidified.

I have expressed earlier my doubt that these changes are necessarily properties of retinene itself, though I saw no better alternative at the time than to ascribe them to this substance (Wald, 1937–38, page 810). Synthetic "retinene₁" however does not change at all in spectrum with pH. What is stranger, neither does natural retinene₁ following adsorption on aluminum oxide. We are exploring these relationships further.

IV

The 545 mμ Chromogen

Spectrum.—The spectrum of the 545 mμ chromogen in chloroform is shown in Fig. 2. The main absorption band lies in the near ultraviolet at about 380 mμ, and a secondary maximum appears at about 290 mμ.

With change of solvent the spectrum undergoes the same peculiar type of displacement as is found in the retinenes. This is shown in Fig. 3. In absolute ethanol the band maxima lie at about 376 and 290 mμ, in hexane at about 361 and 277 mμ.

When mixed with antimony chloride reagent this substance yields an immediate deep purplish red product, the spectrum of which also is shown in Fig. 2. It possesses the main maximum at about 545 mμ by which the substance is temporarily designated. A secondary maximum appears at about 328 mμ. The latter lies very close to the position of the absorption band of vitamin A₁ in chloroform. It probably means that the antimony chloride product includes a conjugated polyene system of the same length as is found in vitamin A₁ (5 conjugated double bonds) in addition to the more extended system responsible for the main band.

The 545 mμ chromogen does not fluoresce, either in solution, or in the adsorbed condition.

Acid-Base Properties.—From the mode of formation of the 545 mμ chromogen

one might suppose it to be a higher oxidation product of vitamin A_1 than the aldehyde, perhaps therefore vitamin A_1 acid.   Its properties, however, do not
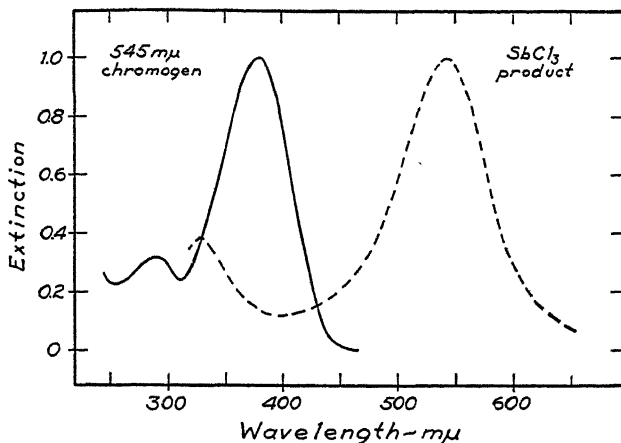
FIG. 2. Absorption spectra of the 545 mμ chromogen in chloroform, and of its wine-colored product with antimony chloride.
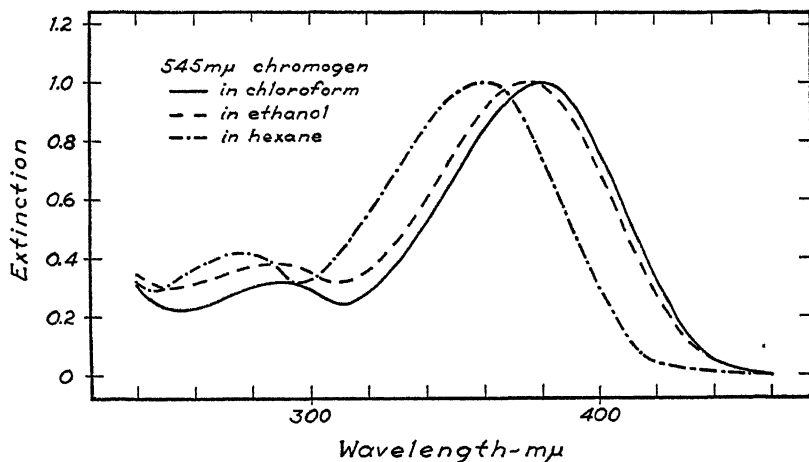
FIG. 3. Absorption spectra of the 545 mμ chromogen in chloroform, ethanol, and hexane.   The spectrum displays a peculiarly large displacement between hexane and polar solvents, due apparently to the presence of a conjugated carbonyl group.

agree with those reported for vitamin A_1 acid (Arens and Van Dorp, 1946; Karrer, Jucker, and Schick, 1946); nor does it display any acidic character whatever.

On the contrary this substance behaves in solubility like a weak base, com-

parable in this with a nitro- or chloroaniline. It is taken up strongly by mineral acids with the formation of highly colored products. Concentrated sulfuric acid removes it completely from hexane solution, forming a salmon-colored product with an absorption maximum at about 520 mμ. Concentrated hydrochloric acid (37 per cent) also extracts it completely from hexane, forming an unstable orange-red product with a maximum at about 500 mμ. Dilutions of hydrochloric acid down to about 27 per cent still extract perceptible color from hexane solutions of the pigment. Still higher dilutions of acid remain uncolored, but a purplish red material forms in the acid-hexane interface.
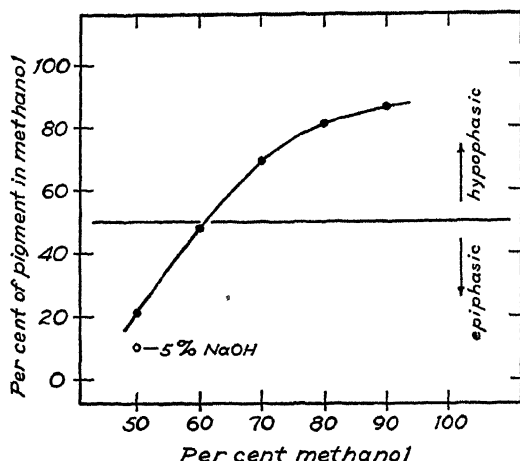
FIG. 4. Partition of the 545 mμ chromogen between petroleum ether and various concentrations of aqueous methanol. The extinctions of pigment in both layers have been added together, and the percentage of the total extinction found in the methanol layer is plotted as ordinate.

The 545 mμ chromogen shares this mildly basic character, as also the formation of highly colored products with mineral acids, with a number of carotenoid pigments, all of which represent higher levels of oxidation than the dihydroxy-carotenoids: flavoxanthin, $C_{40}H_{56}O_3$; capsanthin, $C_{40}H_{58}O_3$; capsorubin, $C_{40}H_{60}O_4$; violaxanthin, $C_{40}H_{56}O_4$; azafrin, $C_{27}H_{38}O_4$; fucoxanthin, $C_{40}H_{56}O_6$. This is a first indication, therefore, that the 545 mμ chromogen may be a polyoxy-carotenoid.

Partition.—The 545 mμ chromogen is extraordinarily hypophasic. In partition between petroleum ether and various dilutions of methanol, it shows very great tendency to enter the methanol layer. The quantitative results of a graded series of such partitions are shown in Fig. 4. In each case the pigment was distributed by violent shaking between equal volumes of petroleum ether

and aqueous methanol.   The amounts of pigment entering each layer were determined by measuring the maximal extinctions, at 360 mμ in petroleum ether, and at 375 to 385 mμ in the various concentrations of methanol.   The figure shows the percentages of total pigment entering the methanol layer.

These measurements show that the 545 mμ chromogen is hypophasic even with 65 per cent methanol.   It distributes about equally between petroleum ether and 60 per cent methanol.   With 50 per cent methanol about 20 per cent still enters the methanol layer; and this fraction is approximately halved with 5 per cent sodium hydroxide in 50 per cent methanol, showing the absence of acidic character.

The 545 mμ chromogen therefore is as highly hypophasic as the most hypophasic of the carotenoid alcohols.   In its partition behavior it resembles fucoxanthin, a C_{40} carotenoid possessing 4 to 5 hydroxyl groups, and probably 1 or 2 carbonyls.

Adsorption.—Like the higher carotenoid alcohols also, the 545 mμ chromogen is strongly adsorbed on calcium carbonate.   On columns of this substance it is adsorbed close to the top as a rather diffuse yet homogeneous yellow zone, which moves downward extremely slowly on prolonged washing with petroleum ether.   With mixtures of petroleum ether and benzene (C_6H_6) 1:1 to 1:3, the pigment moves at moderate speed down the column, becoming increasingly diffuse, yet remaining a single zone.

Photosensitive Derivatives.—Carotenoids—including the retinenes and vitamins A—quite generally form with antimony chloride highly unstable colored products which fade rapidly.   The wine-colored product formed by the 545 mμ chromogen, however, is quite stable, at least for a period of hours.

This product displays a curious and I believe significant property. *It is highly photosensitive.*   In intense light it bleaches in 1 to 2 minutes to an almost colorless condition.

The course of such a bleaching experiment is recorded in Fig. 5.   The antimony chloride product is originally stable in darkness.   On exposure to a moderately bright light, it bleaches partially (solid lines).   It appears to recover in part in subsequent periods in darkness (broken lines).   It is clear from this experiment that the antimony chloride product displays a high order of light sensitivity.

The colored products which the 545 mμ chromogen yields with sulfuric and hydrochloric acids are also markedly photosensitive.   The salmon-pink sulfuric acid product bleaches to yellow-orange, the maximum shifting from 520 mμ toward shorter wavelengths.   The orange-red hydrochloric acid product, as has been remarked, is highly unstable even in darkness; but on exposure to sunlight it bleaches with enormously increased rapidity, losing virtually all its color within about 15 seconds.

V

General Considerations

Chromatographic Reactions.—The process which has been described for converting vitamin A_1 to "retinene₁" or to the 545 mμ chromogen is of considerable general interest. In such a chromatographic process, one is dealing with a highly oriented type of reaction. The solid, in this case manganese dioxide, is
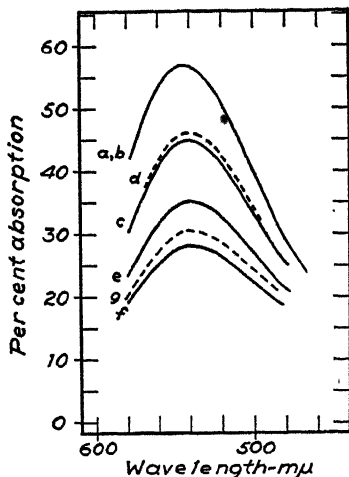
Fig. 5. Bleaching of the 545 mμ antimony chloride product in moderate light. The spectrum of this solution was measured immediately after mixing the reagents (*a*). After 7 minutes in darkness it was found unchanged (*b*). It was irradiated for 30 seconds with a 60 watt tungsten lamp at a distance of about 9 inches (*c*). Left dark for 10 minutes longer, the absorption *rose* to (*d*). It was re-irradiated as before for 1 minute (*e*), then for 1 minute longer (*f*). Left in darkness for another 32 minutes, the spectrum again *rose* to (*g*). The curves shown are tracings of spectra measured with the recording photoelectric spectrophotometer of Hardy.

at once adsorbent and reagent. The adsorbed molecules are attached to it at one or more specific points, and it is presumably at these that the reaction occurs.

One may expect from such an arrangement a high degree of specificity, direction, and control, mimicking on occasion the character of an enzymic process. The products may be expected to be more restricted in number and on occasion different in kind from those obtained in comparable reactions in free solution. From this viewpoint the use of solid adsorbents which react with their adsorbates deserves careful systematic investigation.

It is an instance of the extraordinary insight of Michael Tswett, the founder of chromatography, to have foreseen such a development. In his 1906 paper

he remarks: For special purposes, however, one will turn just to chemically effective adsorbents (hydrolyzing, reducing, oxidizing).

Structure of the 545 Mμ Chromogen.—Determination of the detailed structure of this substance must await its study by degradation, synthesis, and the assay of specific groups. One can gain some insight into its general nature, however, from its mode of origin and the properties already described.

This product of the mild oxidation of vitamin A_1 probably also contains twenty carbon atoms. The displacement of its absorption spectrum toward the red from that of vitamin A_1 indicates a longer conjugated system. For the reasons which follow, it seems probable that this is achieved by adding to the polyene structure of vitamin A_1 a conjugated carbonyl group.

The evidence that "retinene₁," the precursor of the 545 mμ chromogen, contains an aldehydic carbonyl (Ball, Goodwin, and Morton, 1946) provides a

TABLE I

Refractive indices and dipole moments of pentane and hexane (which together effectively constitute petroleum ether), absolute ethanol, and chloroform.

Solvent	Refractive index, n_D	Temperature	Dipole moment
		°C.	Debye units
Pentane............................	1.358	15.7	0
Hexane............................	1.375	20	0
Ethanol...................	1.361	20	1.696
Chloroform....	1.446	20	1.18

first indication of this. But this matter can be approached independently through spectroscopic data, which reflect not only upon the structure of the 545 mμ chromogen but on that of retinene₁.

The spectra of substances in solution ordinarily are displaced toward longer wavelengths with increase in refractive index of the solvent (Kundt's rule). This is true also of carotenoids and synthetic polyenes. On this basis the spectrum should shift little or not at all when a carotenoid is transferred from hexane or petroleum ether to ethanol, which has an almost equal refractive index; but should be displaced markedly on transferring to the much more highly refringent chloroform (Table I).

This is the type of displacement observed in the great majority of carotenoids. Between hexane and ethanol the spectrum is shifted only 0 to 2 mμ, between either of these solvents and chloroform 9 to 15 mμ (Table II).

The vitamins A (A_1 and A_2) behave also in this manner. The retinenes, however, present an entirely different type of relation. In them there is an abnormally large shift of spectrum between hexane and chloroform (about 20 mμ); and the spectrum in ethanol lies close to that in chloroform. Among

carotenoids of known structure this peculiar relation is found also in rhodoxanthin. One finds it again in the 545 mμ chromogen (Fig. 3 above; Table II).

Its significance appears to be as follows. Rhodoxanthin ($C_{40}H_{50}O_2$) possesses two carbonyl groups in conjugation with its polyene system. According to Morton, "retinene₁" also possesses a conjugated carbonyl group. The carbonyl radical is highly polar, and the positive charge on the carbon atom tends to be transmitted down the length of the polyene chain. Such polyene carbonyl compounds therefore exist as resonance hybrids of a number of alternative structural arrangements. In the case of vitamin A₁-aldehyde, for example, the limiting states may be diagrammed as follows:—
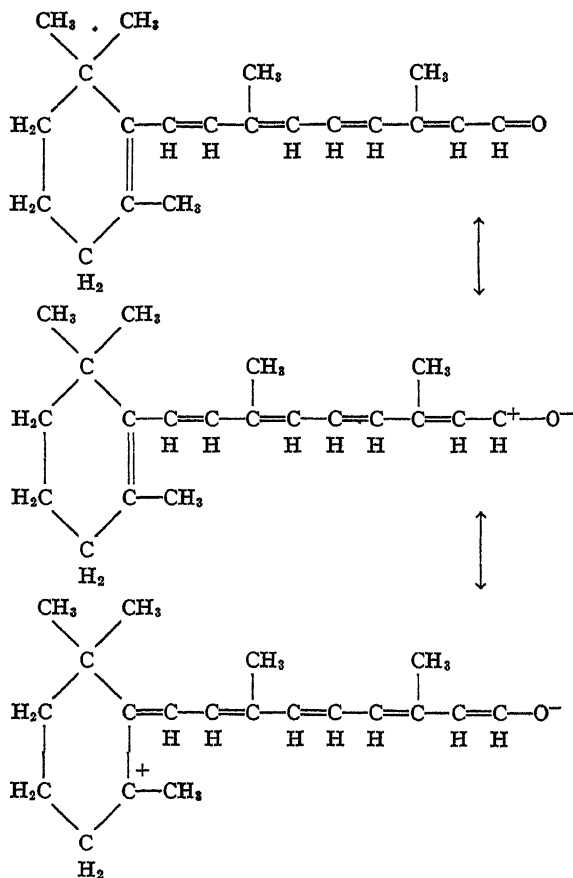
Such resonance hybrids exhibit a special shift of absorption spectrum toward the red.[1] Thus, while the addition of an ordinary ethylene group (—CH ═

[1] A statement of this general relation and references can be found in Pauling (1944), pages 281 and 431.

CH—) to a conjugated polyene chain shifts the spectrum about 20 to 25 mμ toward the red, the addition of a conjugated carbonyl shifts it about 30 to 40 mμ (compare for example the spectra of α- and β-apo-2-carotinols with those of the corresponding aldehydes (von Euler, Karrer, and Solmssen, 1938)). Such a large shift in spectrum (40 mμ in hexane) is found in retinene$_1$ as compared with vitamin A$_1$; and a slightly smaller but comparable shift (35 mμ in hexane) is found in the 545 mμ chromogen compared with vitamin A$_1$.

This special effect of the carbonyl group is enhanced in solvents which are themselves polar. In these one finds *superimposed* upon the spectral changes

TABLE II

Absorption maxima of carotenoids in hexane (or petroleum ether), ethanol, and chloroform showing the displacement of spectrum in a few representative carotenoids, and the special position which such a carbonyl-containing carotenoid as rhodoxanthin shares with retinene$_1$ and the 545 mμ chromogen. When the spectrum has multiple bands, that of longest wavelength is given. The column II − I shows the shift of spectrum in mμ between hexane and ethanol, the column III − II that between chloroform and ethanol.

Carotenoid	I Hexane	II Ethanol	II − I	III Chloroform	III − II
α-Carotene................................	478	476	−2	485	9
β-Carotene................................	482	482	0	497	15
Cryptoxanthin..	484	486	2	497	11
Lutein......................................	476	476	0	488	12
Violaxanthin.............................	472	472	0	484	12
Vitamin A$_1$................................	325	325	0	332	7
Rhodoxanthin..........................	524	538	14	546	8
Retinene$_1$................................	365	380	15	387	7
545 mμ chromogen......................	360	376	16	380	4

which go with refractive index of the solvent a special effect correlated with the dipole moment of the solvent. While hexane and ethanol are very similar in refractive index, in dipole moment ethanol comes close to chloroform, and is far removed from the homopolar hexane or pentane (Table I).

In carotenoids which contain a conjugated carbonyl group, therefore, the displacement of spectrum between hexane and chloroform is abnormally large; and the spectrum in ethanol tends to approach that in chloroform. This type of behavior in the retinenes and the 545 mμ chromogen is *prima facie* evidence of the presence of such a conjugated carbonyl group.[2]

[2] It has been noted a number of times that carotenoids and synthetic polyenes which contain carbonyl groups in conjugation with the polyene system exhibit peculiarly large changes in color or spectrum on transfers between hexane and alcohol (Zechmeister and von Cholnoky, 1935; Hausser, Kuhn, Smakula, and Hoffer, 1935; Zechmeister and Tuzson, 1936).

The further properties of the 545 mμ chromogen indicate the presence of hydroxyl groups. Its highly hypophasic character in partition between petroleum ether and aqueous methanol, its strong adsorption on calcium carbonate, and its mildly basic character and production of colored products with mineral acids, all range it among the higher carotenoid alcohols. Its behavior in all these situations is comparable, for example, with that of fucoxanthin, a C_{40} carotenoid containing 4 to 5 hydroxyl groups and probably 1 or 2 carbonyls.

What is decisive in such structures is probably the ratio of polar groups to the length of hydrocarbon chain. From this viewpoint the similarity in behavior to fucoxanthin would suggest in such a C_{20} carotenoid as the 545 mμ chromogen the presence of two hydroxyls. This inference is consistent with the observation that in those properties which seem to depend upon hydroxyl groups, the 545 mμ chromogen clearly surpasses vitamin A_1, which possesses a single hydroxyl.

Considerations such as these can never provide more than a guide to further study; but the net result of this argument is that there is good reason to believe the 545 mμ chromogen to be a hydroxy-carbonyl derivative of vitamin A_1, which probably contains two hydroxyls in addition to one carbonyl group.

Relation to Visual Photopigments.—From the viewpoint of visual chemistry, it is obviously a stimulating observation that from vitamin A_1, the natural precursor of rhodopsin, one can obtain synthetic derivatives which bear some resemblance to the photosensitive pigments of the retina in spectrum, and which are themselves light-sensitive. Such are the products of the action of antimony chloride or mineral acids upon the 545 mμ chromogen. They have a more general status, which I shall discuss in a later paper.[3]

I think it very probable that in such products one approaches closely the fundamental structures of the visual photopigments; and that the essential process in their formation is the union of two such molecules as the 545 mμ chromogen—or indeed the retinenes—with the loss of water.[4] This matter also must be reserved for a later paper.

[3] *Note added in proof.*—After this was written I found that the blue products of the action of antimony chloride on retinene₁ (natural or synthetic) and on vitamin A_1 also are light-sensitive. These products break down even in the dark, that from retinene₁ slowly, that from vitamin A_1 rapidly. In both cases light greatly speeds the dissipation of the blue color. This action of light on the vitamin A_1-antimony chloride product has been reported earlier by Caldwell and Parrish (*J. Biol. Chem.*, 1945, **158**, 181). As these authors note, in using the antimony chloride reaction for the quantitative estimation of vitamin A_1, the photosensitivity of the blue color makes it important to use a photometer or colorimeter which is sparing of light. This precaution must also be extended to retinene₁.

[4] Meunier and Vinet (1945) have suggested that a yellow pigment obtainable from vitamin A_1 on chromatographing on calcium hydroxide or on certain acid earths, or on treatment with antimony chloride, is di-vitamin A_1 ether, the product of condensing two molecules of vitamin A_1 with elimination of water.

SUMMARY

Ball, Goodwin, and Morton (1946) have reported that vitamin A_1 in contact with solid manganese dioxide is transformed slowly into a substance which displays spectroscopic properties of retinene$_1$. The latter is known to be the precursor of vitamin A_1 in the rhodopsin cycle of the retinal rods. The synthetic product is here referred to as "retinene$_1$."

In the present experiments this observation is confirmed. The procedure is recast in the form of a chromatographic oxidation. Manganese dioxide is packed in a column, vitamin A_1 solution poured in at the top, and the product drawn off in the filtrate. Depending upon the proportions of manganese dioxide and vitamin A_1, the product is either "retinene$_1$," or a new substance which yields with antimony chloride a wine-red product with maximal absorption at 545 mμ (545 mμ chromogen). This procedure is an example of a potentially important class of chromatographic reactions.

The synthetic "retinene$_1$" is virtually identical with the natural substance in absorption spectrum and antimony chloride reaction. It lacks the pH indicator properties of crude natural retinene$_1$.

The 545 mμ chromogen possesses absorption maxima at *380* and 290 mμ in chloroform; at *376* and 290 mμ in ethanol; and at *361* and 277 mμ in hexane. It is non-fluorescent. It has no acidic character, but on the contrary is mildly basic, being extracted from hexane by sulfuric or hydrochloric acids to form orange-red products. In partition between petroleum ether and aqueous methanol it is highly hypophasic. It is adsorbed strongly on calcium carbonate.

Certain peculiarities in spectral behavior indicate the presence of a carbonyl group in the 545 mμ chromogen, and support Morton's proposal that such a group occurs in retinene$_1$. Other properties of the 545 mμ chromogen indicate hydroxyl groups. This substance therefore appears to be a hydroxy-carbonyl derivative of vitamin A_1.

The red products which the 545 mμ chromogen forms with antimony chloride or with sulfuric or hydrochloric acids are all markedly light-sensitive. They appear to be formed by the condensation of two molecules with loss of water; and to bear a close generic relation to the prosthetic groups of the visual photopigments.

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EVIDENCE OF ACTIVE TRANSFER OF CERTAIN NON-ELECTROLYTES ACROSS THE HUMAN RED CELL MEMBRANE

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In discussions of cellular permeability frequent reference is made to the peculiar sensitivity of some mammalian erythrocytes to small traces of copper, observed in studies of the penetration of glycerol into these cells. The retarding effect of copper on hemolysis of human red cells in isosmotic glycerol solutions was first reported by Jacobs and Corson (1934); Jacobs and his group (1938) have subsequently demonstrated this action in the rat, rabbit, and guinea pig, though not in many other mammals tested. With suitable precautions against interference by extraneous protein, Jacobs and Stewart (1946) detected the inhibition of entry of glycerol into the human red cell by copper at concentrations around 10^{-7} M. With 90 per cent inhibition, the amounts of copper required could not have covered more than about 1 per cent of the total cell surfaces involved; furthermore, no diminution in the effect was found when the cells were quickly removed and replaced from a fresh suspension, so that only a small fraction of even this small amount of copper was actually removed in the inhibitory processes. These considerations clearly suggested a special mechanism for glycerol entry into these cells, apparently localized in limited regions on the cell surfaces. Davson and Reiner (1942), in discussing the active transfer of sodium across the cat erythrocyte membrane, called attention to the evidence then at hand of an analogous active movement of glycerol in the human red cell.

Such small quantities of copper as those found effective in this connection typically show inhibitory effects on certain types of enzymatic reactions (Hellerman, 1937; Rapkine, 1938). Enzymes whose activity depends on readily available sulfhydryl (thiol) groups are often interfered with by small traces of copper, as well as by various other oxidizing agents, and by alkylating or mercaptide-forming agents (Barron and Singer, 1945). Thus it seemed expedient to test the effect on glycerol hemolysis of some of these agents which resemble copper in their inhibitory effects on other systems. The results of these experiments suggested that the entrance of glycerol into the human erythrocyte might involve the activity of a sulfhydryl-containing enzyme or at least of some sulfhydryl groups located at the cell surfaces.

Later experiments in which volume changes were followed by the photo-

metric method developed by Ørskov and Parpart lent further support to this suggestion.   By this means it was also possible to study the rate of penetration of glucose, which enters the human red cell only very slowly, and in isosmotic solutions of which osmotic hemolysis is never attained.   The experiments to be described seem to indicate an active transport, on the part of the cell membrane, of both glycerol and some hexoses, though not by identical mechanisms in the two instances.

Materials and Methods

Human erythrocytes were used throughout; blood was drawn from the antecubital vein and citrated, or by lancet and massage from the finger-tips into a large volume of saline solution.   The cells were washed, in either case, by several successive centrifugations in relatively large volumes of the suspension medium; the final suspensions were refrigerated at about 5°C. until used.   Cells were used for as long as 8 days following their withdrawal from the body, but were always washed several times and resuspended in fresh saline solution on the day of use.   The concentrations of suspension used varied as the plan of the experiments and the details of the apparatus were changed, but were always such that the cells represented a very small fraction of the total volume.   ·

The experiments on hemolysis were performed in pyrex test tubes of 7 ml. capacity; 1 to 3 drops of a cell suspension in saline solution were added to 5 ml. of the solution to be tested, and the time to hemolysis recorded with a stop-watch.   The criterion for hemolysis was the visibility through the tube of a linear source of light; this crude method is more than sufficiently reliable to reveal differences of the order of magnitude of those to be reported.

The photometric cytometer used varied in exact form as the experiments progressed. The light source was a single straight-coil filament headlight bulb operated from a 6.3 volt Sola constant-voltage transformer.   The light passed through a filter of $CaCl_2$ solution, or through the water in a constant-temperature jacket, and thence through a flat sided vessel of 7 to 40 ml. capacity, containing the cell suspension. The central beam passing through the suspension fell on a Weston photronic cell, type 3, model 594 GB, attached to a galvanometer, either Leeds and Northrup type R2500, or Rubicon type L, from which the deflection was observed on a frosted plastic scale.   All experiments, except as specifically noted otherwise, were performed at the ambient room temperature.

The saline solution used in the earlier work was simply $M/6$ NaCl, buffered at pH 7.1 with $M/50$ sodium phosphates.   Later, a balanced solution containing small amounts of $CaCl_2$, $MgCl_2$, and KCl, approximately at plasma concentrations, was used; and during one stage of the work, the medium was buffered at pH 6.5 rather than at pH 7.1.   None of these alterations affected the processes studied in any obvious manner, except that the effects of the cupric ion were interfered with in any medium containing appreciable amounts of calcium ion and alkaline phosphate (see Lampitt, Clayson, and Barnes, 1945).

I. GLYCEROL UPTAKE

Results

(a) *Experiments with the Hemolysis Method.*—Significant delays in the hemolysis of human erythrocytes in isosmotic (0.3 M) glycerol solutions (buffered at pH 7.1 with M/50 sodium phosphates) were seen in the presence of Cu^{++}, Hg^{++}, I_2, and p-chloromercuribenzoate. These delays could always be prevented, and in many instances could be reversed, by the addition of suitable quantities of cysteine, glutathione, or other substances containing sulfhydryl or other groups which might compete for the inhibitors with the hypothetical active sulfhydryl groups at the cell surfaces. Typical data are presented in Table I; the concentrations of inhibitors and reactivators shown here are of the order of magnitude of the minimal effective concentrations usually found; though there was considerable variation in this respect, dependent primarily on the time elapsed between the last washing in saline solution and the use of the cells in the test. As noted by Jacobs, sensitivity of the cells to copper is much higher after thorough washing than after the suspension has stood for even a relatively short time, presumably because of the protective effect of small amounts of protein leaking from the cells. This same phenomenon was very evident with respect to the other inhibitors used. The effective concentrations of the reactivating agents were also subject to such variations, and were of course largely dependent on the concentration of inhibitor used. Usually, however, the molecular concentration of the reactivating substance had to equal, or exceed by 1 to 2 times, the concentration of the inhibitor used. Glutathione, cysteine, and thioglycolic acid, each of which contains a readily available thiol group, prevented inhibition whenever present in such concentrations. Alanine was ineffective against I_2 inhibition, and prevented Cu inhibition only at concentrations of about 30 times that of the inhibitor. Ascorbic acid reacted with iodine to prevent its effect on the cells, but had no influence on Cu inhibition. If addition of the sulfhydryl-containing substance·was delayed until sometime after the inhibitor had made contact with the cells, rather than simultaneously with the inhibitor, the prevention of the inhibition was more difficult to accomplish (requiring a higher concentration of reactivator, and bringing about hemolysis less promptly). Instances of this procedure are also included in Table I; in one case (last section of the table) is shown the progressively diminishing effect of cysteine as continued exposure to p-chloromercuribenzoate is allowed. This relation is characteristic also of reactivation of various enzyme systems similarly inhibited (Barron and Singer, 1945).

The most pronounced effects in delaying glycerol hemolysis were obtained with the mercaptide-forming p-chloromercuribenzoate; this agent was there-

fore tested in isosmotic solutions of ethylene glycol, diethylene glycol, and monoacetin, (buffered like the glycerol solutions previously used) to determine

TABLE I

Reversible Inhibition of Osmotic Hemolysis by Glycerol

Inhibitor	Reactivator			Hemolysis time in isosmotic glycerol			
	Nature	Concentration	Time added	Without inhibitor	With inhibitor	With inhibitor and reactivator	
						Total time	Interval
		$M.10^{-5}$	*min.*	*min.*	*min.*	*min.*	*min.*
CuCl$_2$, 10^{-5} M	Thioglycolic acid	2.5	0	1.1	27	1.0	1.0
		4	2			4.4	2.4
	Glutathione	3	0			2.0	2.0
		4	2			4.6	2.6
	Alanine	30	0	1.5	19	1.2	1.2
		30	1			3.3	2.3
	Cysteine	3	0			1.2	1.2
		3	1			2.9	1.9
Iodine, $8 \cdot 10^{-5}$ M	Thioglycolic acid	3.5	0	1.1	90	0.7	0.7
		3.5	2			3.9	1.9
	Glutathione	3	0			1.1	1.1
	Ascorbic acid	2	0			1.1	1.1
	Cysteine	6	0	1.2	85	1.0	1.0
		30	2			2.3	0.3
HgCl$_2$, $8 \cdot 10^{-6}$ M	Glutathione	8	0	1.4	7	0.8	0.8
p-ClHg-benzoate, 10^{-3} M	Thioglycolic acid	400	10	1.9	29	12.7	2.7
	Glutathione	80	10			14.5	4.5
	Cysteine	130	0	2.0	250+	1.7	1.7
			0.3			2.1	1.8
			5			7.0	2.0
			20			24	4.0
			45			53.5	8.5
			180			200	20

whether the inhibitory effect on glycerol hemolysis applied generally to osmotic hemolysis by similar substances.   Specimen results are presented in Table II.

Of the three substances, only monoacetin showed any similarity to glycerol in this respect, and the inhibition of monoacetin hemolysis is not so pronounced as is that of glycerol hemolysis. The process interfered with seems to be moderately specific for glycerol. This parallels the observations of Jacobs and Corson with respect to the Cu inhibition.

Other inhibitors tested failed to affect the rate of glycerol hemolysis; iodoacetate, though a strong inhibitor of many sulfhydryl-containing enzymes by reason of its alkylating reaction with such groups (Dickens, 1933), had no influence on glycerol hemolysis in concentrations up to 0.02 M. Other ineffective inhibitors, tested because of various suggestions, were hydroxylamine, up to $3 \cdot 10^{-3}$ M; NaF, up to 10^{-2} M; NaCN, up to 10^{-2} M; and H_2O_2, up to 1.2 per cent. The arsenical "mapharsen" (3-amino-4-hydroxyphenylarsineoxide hydrochloride), which reacts with some sulfhydryl enzymes to form an inactive

TABLE II

Effect of p-Chloromercuribenzoate on Osmotic Hemolysis by Several Related Penetrating Non-Electrolytes

Test penetrant	Hemolysis time in isosmotic penetrant	
	Without inhibitor	With p-ClHgB, 10^{-3} M
	min.	*min.*
Glycerol	0.7	60
Monoacetin	1.1	3.2
Ethylene glycol	0.3	0.3
Diethylene glycol	0.6	0.6

complex, appeared to inhibit glycerol hemolysis irreversibly at concentrations of 0.2 per cent or more (LeFevre, 1946), but this effect was later attributed to the osmotic protection afforded by the sucrose and Na_2CO_3 with which this drug is mixed in the medicinal ampoules from which it was obtained.

(b) *Experiments by the Photometric Method.*—The use of data based on the timing of hemolysis, in the interpretation of changes in cellular permeability, is always complicated by the possibility that observed differences may reflect changes in fragility or equilibrium conditions rather than actual changes in rate of penetration. Thus it was desirable to check the results of the above experiments by means of some other method which more definitely followed the changes in volume. This was provided in the photometric method developed by Ørskov and Parpart, which had the further advantage that the volume changes of the cells might be followed in media much more nearly normal for the cells than were the isotonic non-electrolyte solutions used with the hemolysis technique.

The inhibitory effects of cupric and mercuric ions, iodine, and p-chloromercuribenzoate upon the rate of entry of glycerol, and the reversibility of this

inhibition by sulfhydryl groups, were thus verified.   Sample records of typical instances of inhibition are shown in Figs. 1 and 2.   The concentrations of the mercaptide-forming inhibitors required to produce inhibition under these circumstances were somewhat higher than those necessary to inhibit hemolysis; these differences were minimized, if not absent, when the non-penetrating non-electrolyte sucrose in isotonic concentration was substituted for the electrolyte medium (Fig. 1).   Parallel tests with thiourea, chosen because its normal rate of entry into the cells is somewhat less than that of glycerol, showed no such inhibition (Fig 2).   Thus the inhibitory effects of these sulfhydryl reagents on glycerol hemolysis is attributable to a real decrease in the rate of entry of glycerol in their presence.

The effect of one particular inhibitor, the glucoside phlorhizin, may be of special interest, since this substance is so specifically active on the transfer mechanisms in the kidney tubule (Walker and Hudson, 1937) and the intestinal mucosa (Nakazawa, 1922; Wertheimer, 1933; Donhoffer, 1935), in which phosphorylation seems definitely to be involved (Lundsgaard, 1933; Laszt, 1935).   The behavior of phlorhizin in delaying the entry of glycerol into human erythrocytes is shown in Fig. 2(b).

Other more or less likely inhibitors without demonstrable effect include mapharsen, alloxan, sodium arsenite, iodoacetate, fluoride, cyanide, maleate, and azide; Parpart, Barron, and Dey (1947) found no effect with p-carboxyphenylarsineoxide, iodoacetamide, iodosobenzoate, or cadmium, but inhibited glycerol hemolysis with chloropicrin.

Discussion

Parpart, Barron, and Dey (1947) suggested that the effect of inhibitors other than copper might be attributable not to interference with a transfer process, but to production of such a change in the hemoglobin structure that the cell is no longer free to alter its volume osmotically in the normal manner. More investigation of this possibility is required; it is evident that exposure of the cells to such agents as p-chloromercuribenzoate, at 10^{-3} M, is not without direct effect on the cell volume, inducing within a few minutes a slight shrinkage (or at least an increase in opacity) which is not apparently reversed upon addition of the reactivators.   This shrinkage is noticeable in several of the figures. Iodine, at inhibitory concentrations, caused discoloration of the hemoglobin. However, the pronounced effects of the inhibitors on rate of volume change in glycerol were not observed with thiourea; and the delay in hemolysis, produced by p-chloromercuribenzoate in isosmotic solutions of glycerol or monoacetin, was not detectable in ethylene glycol or diethylene glycol.   Further, the reversal of inhibition upon addition of substances furnishing sulfhydryl groups indicates that the action of the inhibitors in these experiments is through their effects on such chemical groupings; so that the interpretation of Parpart *et al.*
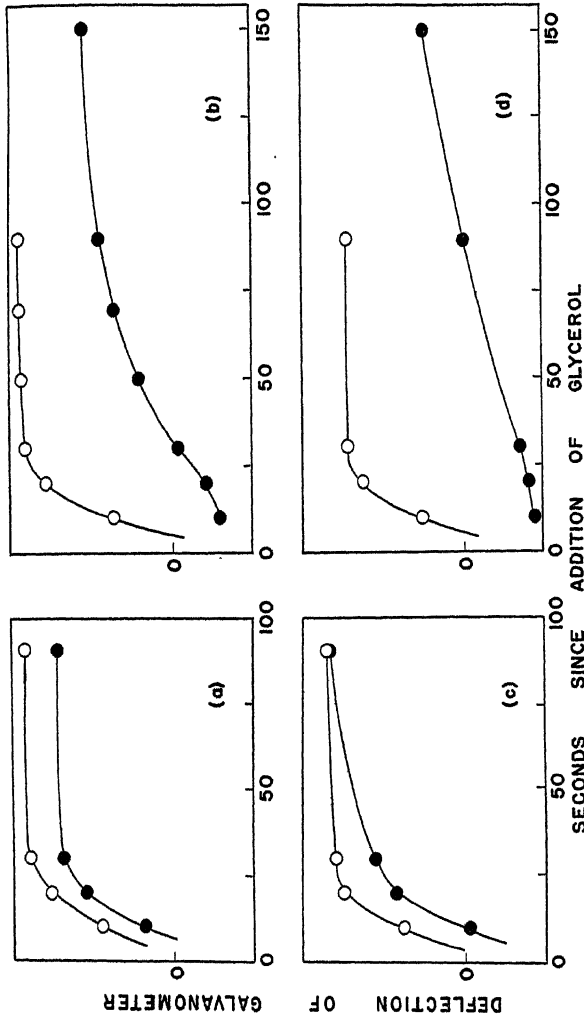
Fig. 1. Inhibition by *p*-chloromercuribenzoate of swelling in glycerol-saline solutions. At zero time, 5 ml. 2.4 M glycerol added to 35 ml. cell suspension.

Solid circles—with *p*-ClHgB,

(*a*) 0.001 M, in isotonic NaCl medium.
(*b*) 0.002 M, in isotonic NaCl medium.
(*c*) 0.001 M, in isotonic KCl medium.
(*d*) 0.001 M, in isotonic sucrose medium.

Open circles—without inhibitor, in same media.

Deflections plotted as movement from position prior to addition of glycerol; upward deflection indicates swelling of cells. Difference between initial and terminal levels results also from dilution of suspension.
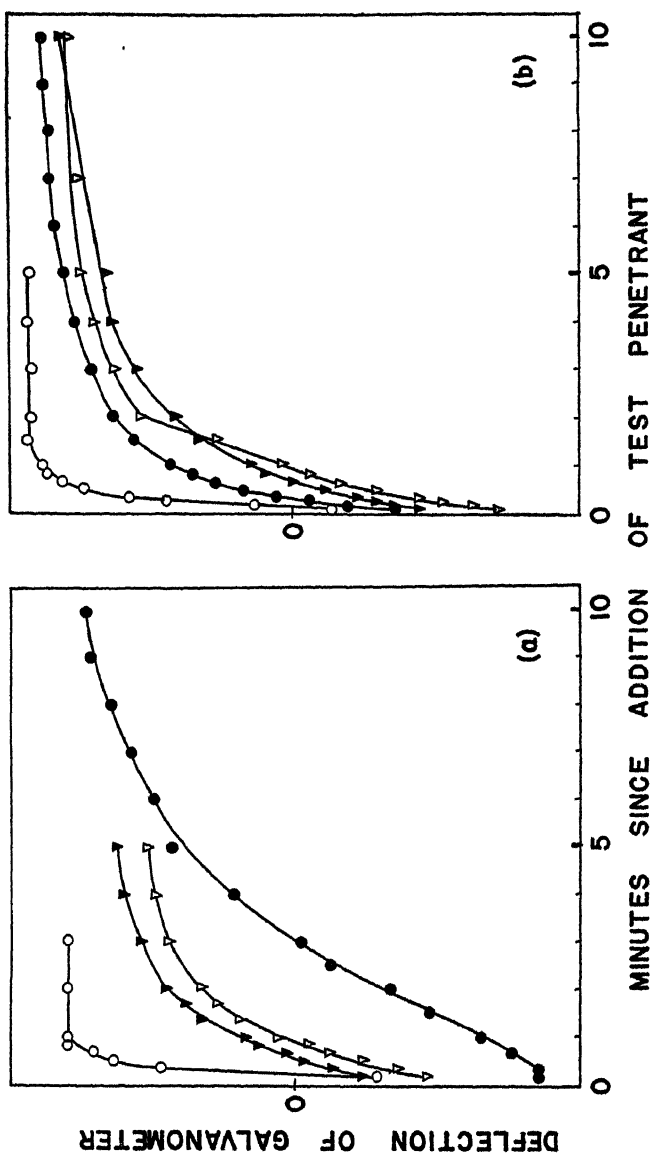
FIG. 2. Inhibition of swelling in glycerol-saline solutions, and absence of inhibition in thiourea-saline solutions. Open symbols—without inhibitor. Circles—glycerol uptake. Solid symbols—with inhibitor. Triangles—thiourea uptake.

(a) At zero time, 5 ml. 2.4 M glycerol or 1.2 M thiourea added to 35 ml. cell suspension in isotonic KCl, with or without p-chloromercuribenzoate, 0.002 M.

(b) At zero time, 1 ml. 1.8 M glycerol or 1.8 M thiourea, in the suspension medium, added to 5 ml. cell suspension in balanced saline medium, with dextrose at 0.05 M, with or without phlorhizin, 0.005 M.

Deflections plotted as in Fig. 1.

would necessitate postulation that the suggested paracrystalline state is readily reversible for some time by this means. Also, as will be reported in the next section, effective inhibitory concentrations of mercuric ion and of p-chloromercuribenzoate in respect to hexose uptake are on the order of 10^{-6} to 10^{-5} M, or 100 to 1000 times more dilute than those used in the experiments
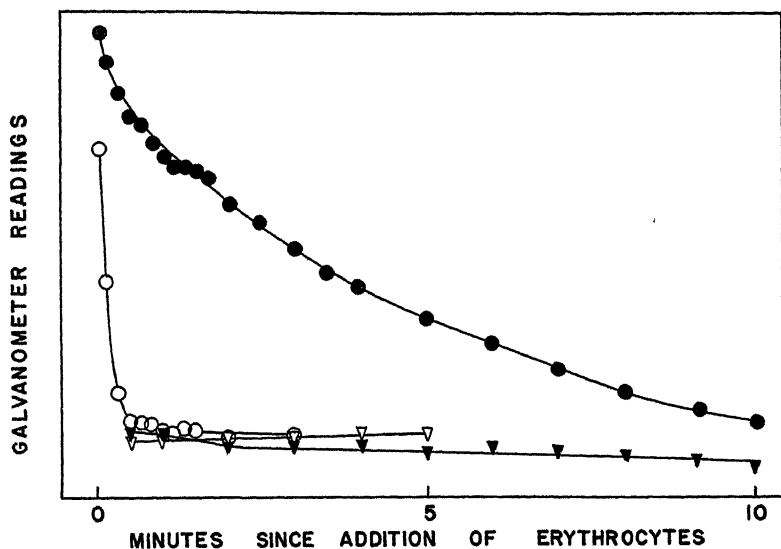
FIG. 3. Inhibition by p-chloromercuribenzoate of exit of glycerol from cell interior.
Circles—cells previously equilibrated with glycerol, 0.4 M, in buffered sucrose, 0.3 M.
Triangles—cells previously in sucrose medium alone.
At zero time, 1 ml. cell suspension added to 5 ml. sucrose medium.
Open symbols—without inhibitor.
Solid symbols—with p-ClHgB, 10^{-3} M.
Identical patterns were obtained with saline medium, but higher concentrations of inhibitor were required, with less pronounced effect, as also with respect to entry of glycerol.

with glycerol, in which the cells at least retain their identity for several hours. And the same inhibition by $CuCl_2$ or p-chloromercuribenzoate of volume changes in glycerol is seen with respect to the exit of this substance from the cell as with respect to its entrance (Fig. 3).

The inhibitory effects of the group of agents used suggested that the transport of glycerol into the human red cell was effected by an active metabolic system in which at least one essential link involved a sulfhydryl group. The pattern of effectiveness and ineffectiveness of the various types of sulfhydryl inhibitors tested indicated further, following Barron and Singer (1945), that

the sulfhydryl groups concerned were of the relatively unavailable type, re-
quiring, except for the peculiar sensitivity to copper, rather drastic or specific
chemical attack to be inhibited.   The most obvious preliminary hypothesis,
by analogy with known instances of active transport of polyhydric molecules
in the kidney tubule and the intestinal mucosa, was that the critical process
involved was a phosphorylation.   Adenosine triphosphate can supply phosphate
to glycerol (Gunsalus and Umbreit, 1945); and the apparent sulfhydryl-con-
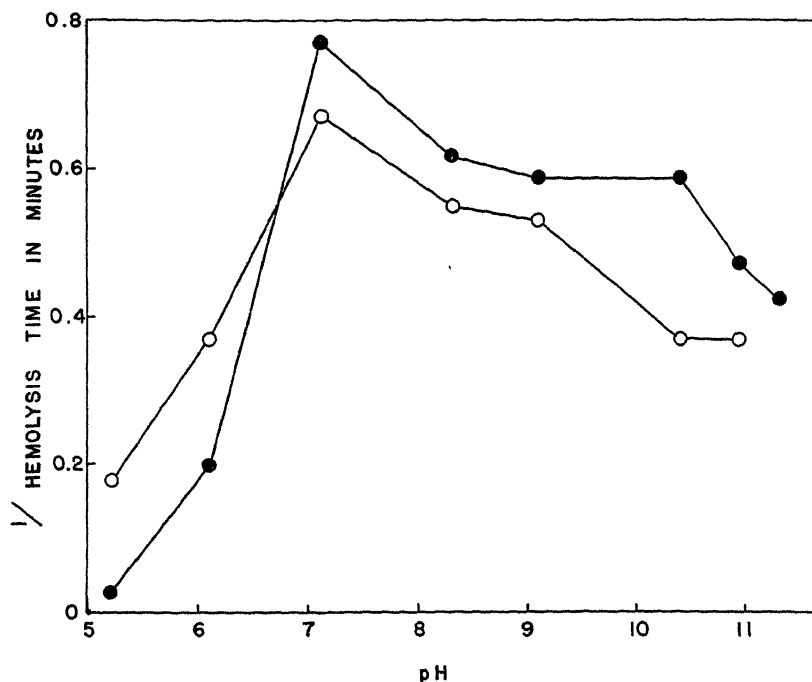
Fig. 4. Influence of pH on rate of attainment of hemolysis in buffered isosmotic
glycerol (solid circles) and monoacetin (open circles).

taining enzyme concerned in these experiments may resemble adenosine tri-
phosphatase.   The latter is not affected by iodoacetate or iodoacetamide
(Needham, 1942), but is reversibly inhibited by copper (Bailey, 1942; Binkley,
Ward, and Hoagland, 1944) and by p-chloromercuribenzoate, though only
questionably by the arsenicals (Singer and Barron, 1944); and is strongly
inhibited by iodine (Ziff, 1944); these inhibitions being reversible by addition
of cysteine, glutathione, or, to some extent, by addition of ascorbic acid.   The
failure of hydrogen peroxide, which prevents activity of ATPase (Mehl, 1944;
Ziff, 1944), to inhibit the glycerol transport mechanism may be attributable
to rapid destruction of the peroxide by red cell catalase.

A further particular in which the activity of the apparent glycerol transport mechanism may be compared with that of ATPase is with respect to the influence of pH. Fig. 4 shows the effect of pH on the rate of attainment of hemolysis in isosmotic glycerol and monoacetin solutions (with sodium phosphate buffer, M/50). These pH-activity curves resemble those of myosin ATPase in showing a pronounced inhibition on the acid side, but differ considerably in the position of the optimal pH; which for myosin ATPase lies in the neighborhood of pH 9 (Bailey, 1942; Singher and Meister, 1945). However, these curves parallel very closely those given by Kalckar (1944) for ATPase from potatoes; and Mehl (1944) describes a second optimum for myosin in the vicinity of pH 7.

Jacobs, Glassman, and Parpart (1935; 1938) have grouped the erythrocytes of a large number of mammalian species into two classes: those which show Cu sensitivity with respect to hemolysis in glycerol, and those which do not. Glycerol hemolysis in the sensitive group generally shares other properties absent in the other class: sensitivity to pH, CO_2, alcohols (Jacobs and Parpart, 1937); a low Q_{10}; and permeability to glycerol disproportionately high in comparison with general permeability. Investigation of the extension of this grouping to include sensitivity to the inhibitors used in the experiments reported here is indicated. Attention might also be directed toward other instances of inordinately high specific permeabilities, as of the mouse red cell for erythritol (Jacobs, Glassman, and Parpart, 1935), and to the *Chaetopterus* egg for glycerol (Lucké, Hartline, and Ricca, 1939).

<div align="center">II. GLUCOSE UPTAKE</div>

<div align="center">*Results*</div>

(a) *Experiments with Inhibitors.*—The use of the photometric method of following volume changes made possible a study of similar osmotic changes in glucose solutions. The procedure generally used was the addition to a cell suspension in isotonic saline solution of an additional 20 per cent of its volume of the same medium containing dextrose at 6 times the final concentration desired, usually isosmotic (0.3 M). The entrance of glucose proved to be as sensitive as that of glycerol to the presence of some of the inhibitors tested. The pattern of effectiveness of inhibitors was somewhat different for glucose and for glycerol. I_2, Hg^{++}, Hg_2^{++}, and *p*-chloromercuribenzoate affect both, but the glucose system is vastly more sensitive to the mercurials. Both are less strikingly inhibited by phlorhizin, at $5 \cdot 10^{-3}$ M; but the volume changes in glucose solutions are entirely unaffected by cupric ion up to $2 \cdot 10^{-5}$ M, much more than is required to effect a great delay in the swelling in glycerol solutions. Other inhibitors found ineffective on glucose uptake included alloxan, mapharsen, lead, iodoacetate, and arsenite. Fig. 5 shows typical instances of inhibition of swelling in glucose solutions by *p*-chloromercuribenzoate in con-

centrations of $2 \cdot 10^{-6}$—10^{-5} M, and a case of almost complete reversal of inhibition, by addition of cysteine shortly after application of the inhibitor, is given in Fig. 6.
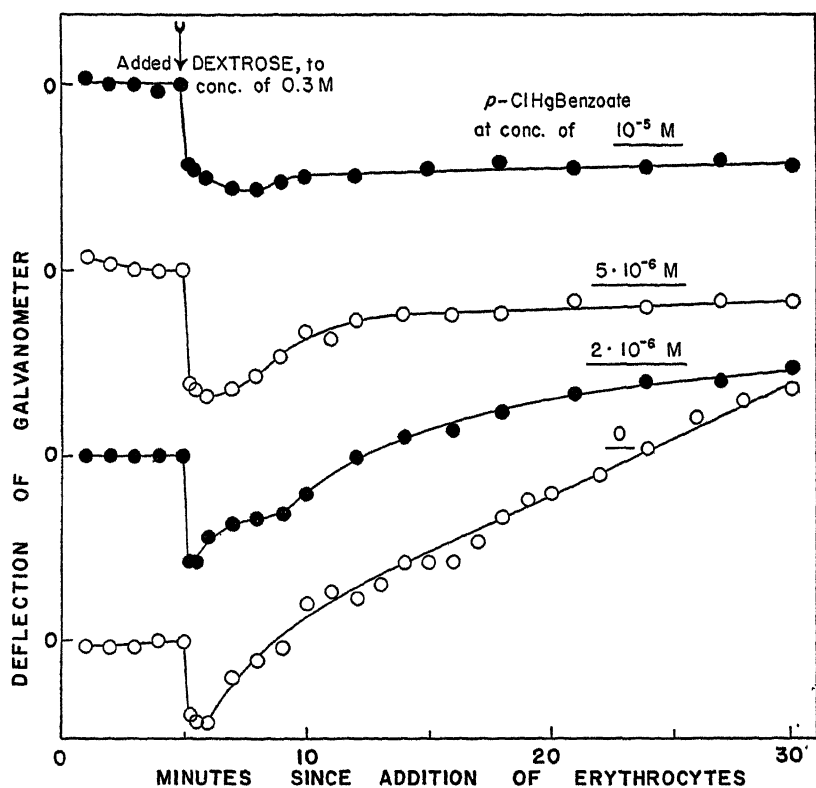
FIG. 5. Inhibition by p-chloromercuribenzoate of volume changes in glucose-saline solutions. Five minutes after exposure of cells to inhibitor in saline medium, at indicated concentrations, added 1 ml. of medium with dextrose, 1.8 M, to 5 ml. of cell suspension. Deflections plotted as in Fig. 1, as movement from position just prior to addition of glucose.

(b) *Experiments on Kinetic Relations.*—Aside from the action of inhibitors, there was reason to suspect that glucose was being carried into the cells actively, on the basis of the dynamics of the volume changes observed. Fig. 7(a) shows the shape of the curves relating cell volume and time following addition of various increments of dextrose to suspensions of cells in isotonic balanced saline solution. Note that equilibration is more rapid the less dextrose added, and that, with the larger additions, the initial rate of volume change increases as the concentration of sugar is decreased. Note also that the general form of the volume changes is a straight line when larger concentrations are in-

volved, but approaches the more usual exponential form with smaller concentrations. Qualitatively, this is exactly the set of relations that would be expected if there were an absolute limit to the amount of glucose that could enter
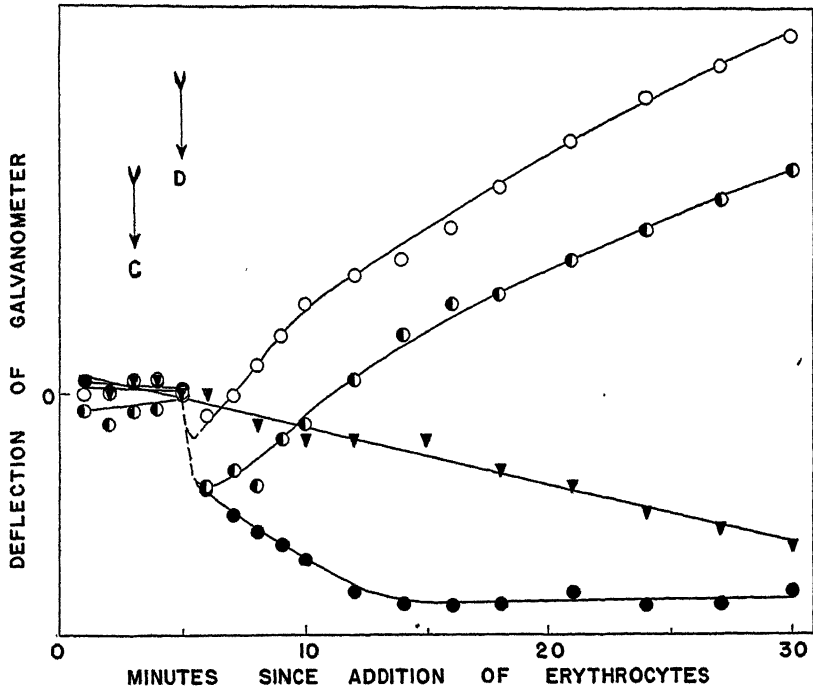
FIG. 6. Inhibition by HgCl₂ of uptake of glucose; reversal of inhibition by cysteine Five minutes after exposure of cells to inhibitor in saline medium (at *D*), add ed 1 ml of medium with dextrose, 1.8 M, to 5 ml. of cell suspension.

Triangles—control with HgCl₂, 10⁻⁶ M, without addition of dextrose.

Open circles—control without HgCl₂.

Solid circles—with HgCl₂, 10⁻⁶ M.

Half-solid circles—with HgCl₂, 10⁻⁶ M; at 3 minutes, (*C*), added one drop of medium with cysteine dihydrochloride to final concentration of 2.5 · 10⁻⁴ M.

Deflections plotted as in Fig. 5.

the cell in a given time, regardless of the concentration gradient. The pattern found does not of course necessarily imply an active transfer mechanism, but would be a likely result of the functioning of such a mechanism.

At any rate, the pattern of these relations is clearly not compatible with the assumption of a simple unhindered passive diffusion process, following Fick's law:

$$\frac{dS}{dt} = kA\left(C_s - \frac{S}{V}\right),$$
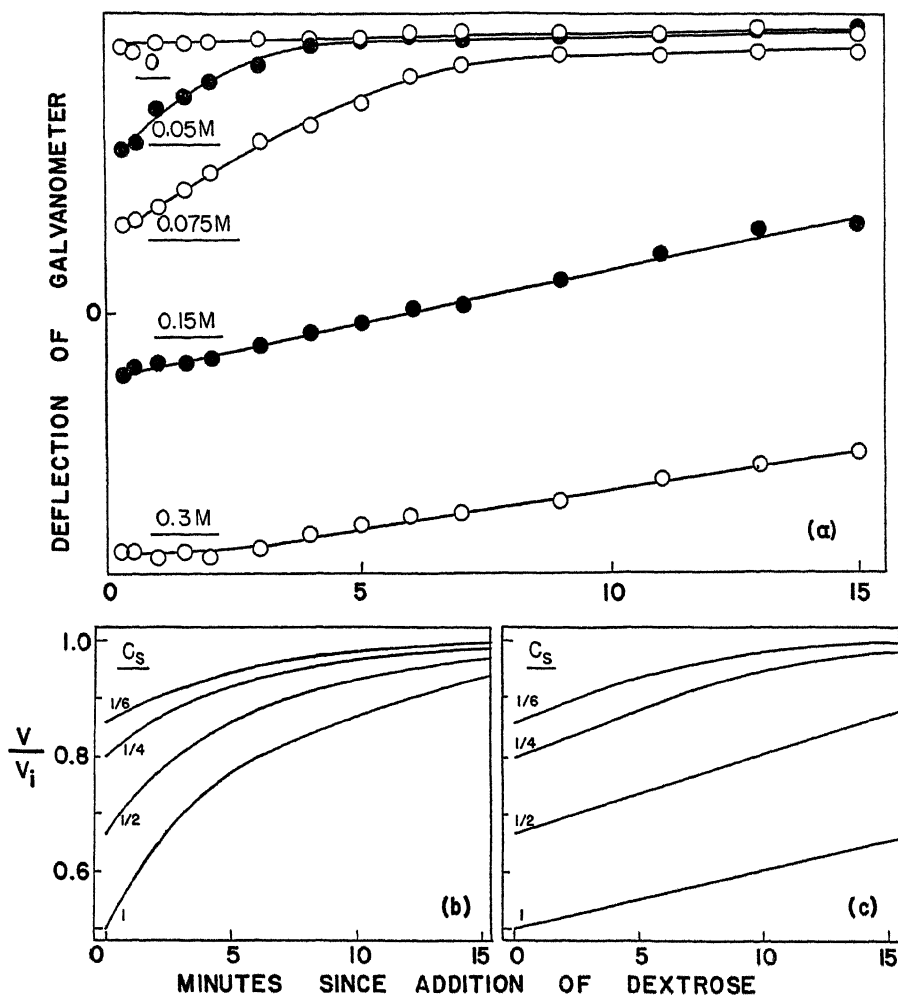
FIG. 7. Kinetics of swelling in glucose-saline solutions.

(a) At zero time, 1 ml. saline medium with dextrose at 6 times final concentration indicated added to 5 ml. cell suspension in medium.   Deflections plotted as in Fig. 1.

(b) Predicted relations in similar experiment, assuming passive diffusion, with $dS/dt = k\ (C_s - S/V)$, with $k = 0.2$ iso-volumes per minute.

(c) Predicted relations in similar experiment, assuming diffusion rate is limited to maximal value, m, ($= 0.02$ iso-content per minute,) by process involving cellular component.

where S is the amount of the penetrating substance within the cell, k is a "permeability constant," A is the area across which the permeation occurs, C_s is the

external concentration of the penetrating substance, and V is the volume of the intracellular fluid in which S is dissolved. Since the cells make up only a small fraction of the total volume of fluid in these experiments, C_s may be regarded as constant. And since the manner in which A varies with V under these conditions in a cell having the form of an erythrocyte is at best uncertain, and since in other instances the area of these cells seems to remain constant in spite of volume changes, the factor A is commonly included in the permeability constant k. In the present instance, the penetrating substance can enter the cell only much more slowly than water, so that the osmotic pressure on the two sides of the membrane may be considered to be essentially identical at all times. Thus,

$$\frac{dS}{dV} = C,$$

where C is the total extracellular concentration, in osmotic units. Hence,

$$\frac{dt}{dV} = \frac{CV}{kC_i(V_i - V)},$$

where C_i is the external concentration of non-penetrating substances (in these experiments, isotonicity), and V_i is the volume of the intracellular fluid in an isotonic medium. Then

$$t = \frac{V_i}{k}\left[1 - \frac{CV}{C_i V_i} + \frac{C}{C_i}\ln\frac{C_s V_i}{C(V_i - V)}\right],$$

with initial conditions as in these experiments; with conditions at isotonicity as the units, this may be written

$$t = \frac{1}{k}\left[1 + (1 + C_s)\left(\ln\frac{C_s}{(1 - V)(1 + C_s)} - V\right)\right].$$

This relation gives a pattern of volume-time curves, with various values of C_s, such as those presented in Fig. 7(b). The behavior of the cells in the glucose solutions clearly cannot be harmonized with this pattern of passive diffusion.

If, however, it be assumed that the transfer involves temporary formation of a complex with some constituent of the cell membrane, or some other type of reaction limited by the quantity available of some such constituent, the simple diffusion will be complicated by this consideration. There may then be some limiting value, m, which dS/dt cannot exceed, regardless of the concentration gradient. This relation gives a pattern of volume-time curves such as that seen in Fig. 7(c). The form of these curves is qualitatively very similar to the observed behavior of the cells in glucose solutions. Quantita-
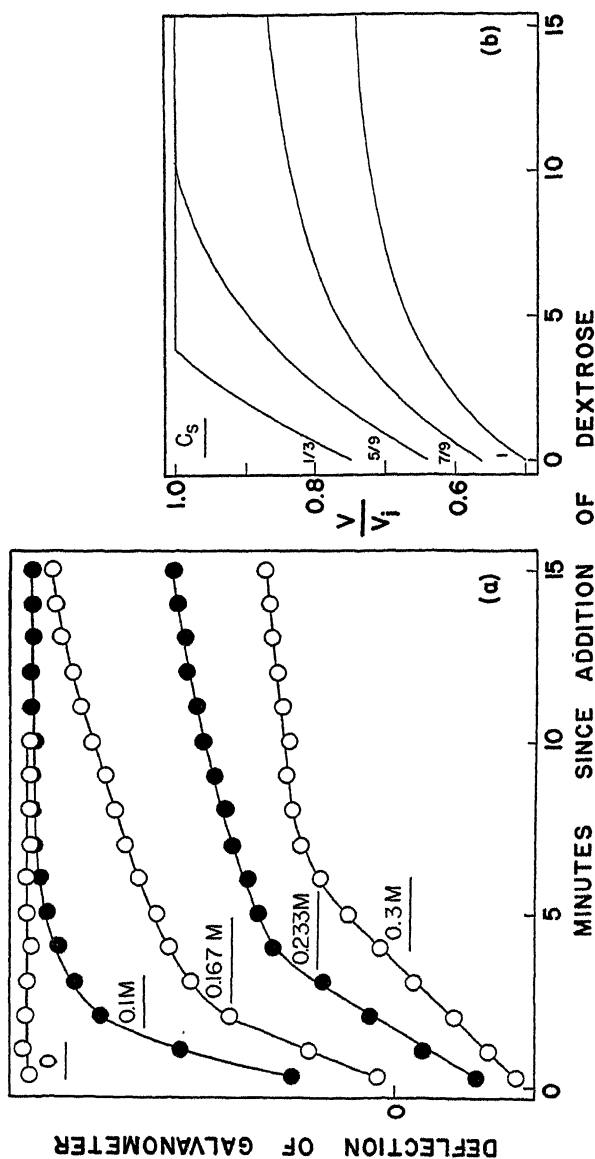
FIG. 8. Kinetics of swelling in glucose-saline solutions.

(a) As in Fig. 7(a), except that temperature here is 37°C.

(b) Predicted relations in similar experiment, assuming transfer rate proportional to degree to which "limiting concentration" has not been attained, with $dS/dt = k(L - S/V)$, with $k = 0.2$ iso-volume per minute, and $L = \frac{2}{3}$ isotones; terminating swelling at moment of attainment of even distribution of glucose.

tively, however, it is impossible, by this assumption, to account for the magnitude of the differences observed in initial rate, dV/dt, with different concentrations of dextrose. For this reason, and because of a further complication in the experimental curves, a more complex relation governing the course of the volume changes was suggested.
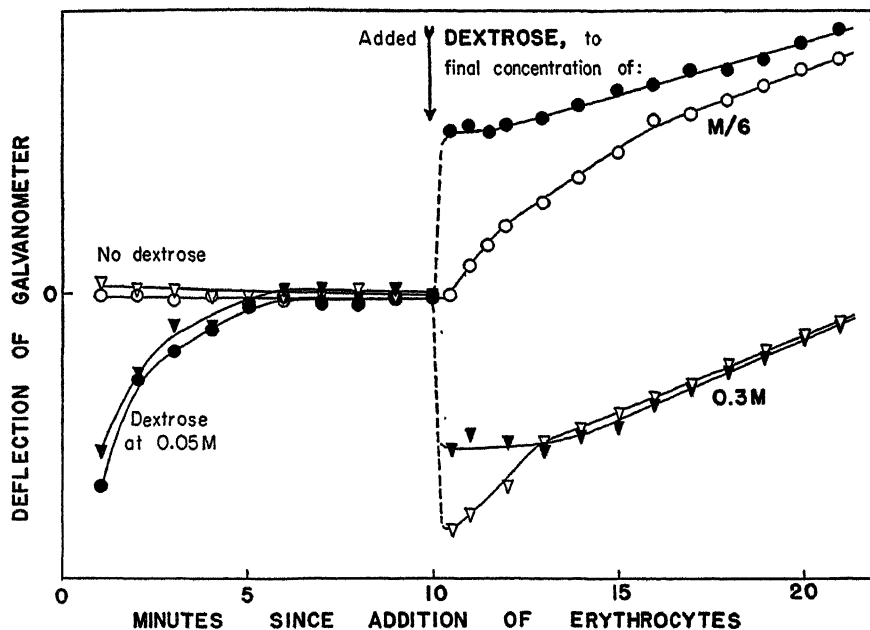
FIG. 9. Effect of previous equilibration with dilute glucose solutions on swelling in glucose-saline solutions. At zero time, 5 ml. cell suspension added to 5 ml. of medium.
Open symbols—without dextrose.
Solid symbols—with dextrose, final concentration 0.05 M.
At 5 minutes, added 2 ml. medium with dextrose, to made final concentration of 0.3 M (triangles), 0.167 M (circles). Deflections plotted as movement from reading just prior to final addition of glucose.

The additional complication is demonstrated in Fig. 8(a); with longer experiments than those shown in Fig. 7(a), or with increased temperatures, it is seen that the initial swelling in the more concentrated solutions slows down rather abruptly after a time, and that this effect is more pronounced the stronger the solution. In some instances, the swelling appears nearly to cease altogether while the cell volume is still considerably less than in an isotonic solution. Furthermore, as shown in Fig. 9, preliminary equilibration with dextrose at concentrations in the neighborhood of what appears to be the intracellular limit, 0.05 to 0.1 M at room temperature, markedly depresses the subsequent

rate of swelling during the initial period, but does not affect the later rate after the process has slowed down.   Thus, it is suggested that the postulated carrier mechanism functions only when the intracellular concentration of glucose is below this critical level.   This assumption might simply be added to either of the two previous suggestions, so that the theoretical curves would show the terminal levelling found experimentally; but this would not improve the fit of the initial stages of the curves.   These compound assumptions may be replaced by a single new hypothesis, the predictions of which approach the experimental curves; this suggestion is: that the rate of transfer of glucose into the cell is proportional to the disparity between the intracellular concentration of glucose and some "limiting" concentration.   Thus the rate of uptake would be independent of the gradient across the membrane (except that thus far no evidence of transport *against* a gradient has been observed).   This relation may be written,

$$\frac{dS}{dt} = k\left(L - \frac{S}{V}\right),$$

where L is the "limiting" intracellular concentration for glucose.   Then, again with isotonic conditions as the units,

$$t = \frac{1 - V(C_s + 1)}{k(C_s + 1 - L)} + \frac{C_s + 1}{k(C_s + 1 - L)^2} \ln \frac{L}{(C_s + 1)(1 - V[C_s + 1 - L])}.$$

This relation, as amended by the limitation that transport against the gradient does not occur, is presented in Fig. 8(b).   This parallels the experimental pattern in the attainment of terminal volumes short of the isotonic volume, and in that the initial rate of swelling decreases with increasing dextrose concentration.   However, aside from quantitative discrepancies, the early stages of the experimental curves appear to be much more nearly straight lines than would be predicted from this relation.   Also, the terminal subnormal cell volumes under most circumstances continue to increase slowly; this may be simply the result of the steady glycolytic activity, the extent of which in these conditions has not been determined.   A more detailed analysis of the applicability of various quantitative hypotheses concerning these relations will be taken up in a later report.   Extension of the system proposed by Shannon (1939) for the transfer of glucose across the kidney tubule appears to be recommended: extracellular glucose in ready equilibrium with its combination product (with some membrane constituent present in constant limited amount), the slower decomposition of this combination at the interior depending on some activity which is suppressed in proportion to the increasing glucose concentration.

(c) *Accessory Experiments.*—Some attempts have been made to check by chemical analytic methods the interpretation of the volume changes followed

in the photometric apparatus. The rapid uptake of glucose from an isosmotic solution in the saline medium, to an intracellular concentration of less than 50 per cent of the external concentration, followed by almost no subsequent change, was verified by analysis of the glucose content of the medium. The inhibitory effects of the mercuric ion were also indicated in this manner; but the necessity of using very dense suspensions of cells, so that the cell glucose uptake would be reflected in a lowering of the extracellular concentration, entailed use of much higher concentrations of mercuric ion than in the other methods. This involved increased initial cell damage by the poison, and perhaps little accord should be given the results thus obtained.

The possibility that phosphorylation might be involved in the transport into the red cell of either glycerol or glucose or of both led to a brief investigation of the possibility that inorganic phosphate might be consumed in the process (incorporated into the organic phosphate carried into the cell interior). Large quantities of human erythrocytes were thoroughly washed in the usual suspension medium, and the suspension finally brought to about 40 per cent. 1 ml. of glycerol or 1 gm. of glucose was added to 80 ml. of such a suspension, and the cells removed by centrifugation after a few minutes. Under these circumstances, the concentrations of phosphate remaining in the supernatant medium were identical with that of the original medium (within the experimental error of about 1 per cent), whether glycerol or glucose or neither had been added to the cells. If even distribution had taken place, as would be expected from the other experiments, about 0.5 gm. of glycerol, or 0.4 gm. of glucose must have been intracellular in these suspensions, neglecting the cell dead space. This would amount to about 5.5 mM of glycerol or 2.2 mM of glucose; but the entire 80 ml. of suspension contained less than 2 mM of inorganic phosphate, assuming equal distribution intra- and extracellularly; and this amount was not changed measurably by the transport. Thus it is evident that phosphate is not fixed by the processes involved in carrying glucose or glycerol into the cell. However, this evidence does not preclude the possibility of a temporary phosphorylation during the transit of the membrane, such as is postulated in the intestinal and renal transfer of some sugars; in fact, Wilbrandt and Laszt (1933) report similar evidence of no change in the intestinal epithelial content of hexose phosphate during active absorption as compared to starvation. This reversible sort of transfer system would also be indicated by the fact that the inhibitors delay the process in both directions.

Discussion

The anomalous behavior of glucose in entering the human erythrocyte has occasioned earlier comment; Klinghoffer (1935) reported rapid equilibration between cells and plasma when small amounts of the sugar were added to blood, yet the cells could be kept in isosmotic glucose almost indefinitely with-

out hemolysis. Klinghoffer set the concentration of about 2 per cent as the critical range, below which even distribution between cells and plasma is rapidly attained, and above which an extracellular excess is maintained almost indefinitely. This figure was also indicated by the observation that human red cells in isosmotic glucose solutions swell rapidly to about 140 per cent of their original volume, then much less readily.

Bang and Ørskov (1937) found that the permeability constant for glucose in these cells was reduced by about 60 per cent by doubling the external concentration of the sugar in the neighborhood of 0.05 M. These authors refer to theses by Ege and Bjering as the first reports on this anomaly; Bjering apparently also observed that the entry of glucose into the cell is prevented by $Hg(CN)_2$, and that the normal uptake is at first rather rapid, later much slower. Meldahl and Ørskov (1940) checked this latter point in finding a progressively diminishing permeability "constant" as the entry of glucose proceeded, as calculated from the volume-time relations.. The constants for thiourea, glycerol, and malonamide, on the other hand, appeared in similar experiments to increase progressively; this was interpreted as indicating increased pore size with swelling of the cells. In the calculation of these constants, Meldahl and Ørskov applied a form of Fick's law which assumes a constant cell volume, although changing figures for the volume were substituted in the equation; this introduces considerable error. However, the oversight leads to an apparent decrease of the "constant" with time, so that the observed discrepancies iin the case of glycerol, thiourea, and malonamide are even greater than reported. With glucose uptake, the marked progressive decrease in the "constant" is not so great as originally calculated, but is still very pronounced; so that the conclusions drawn by Meldahl and Ørskov are qualitatively valid. The behavior in glucose solutions was attributed by these observers to adsorption of glucose on the cell membranes, blocking passage through the pores. Some such process may well be involved, but direct evidence is lacking. Wilbrandt, Guensberg, and Lauener (1947) recently showed that the "permeability constant" for glucose in the human red cell may be more than 1000 times larger in a dilute solution of glucose than in a concentrated solution.

The investigations of Cori, Lundsgaard, Wilbrandt, Verzár, Laszt, and others on the absorption of sugars from the intestinal lumen indicated that there was some degree of specificity in selection of sugars to be moved across the intestinal lining by means of a phosphorylating mechanism. Wertheimer (1933) reported inhibition by phlorhizin of the uptake from the rodent intestine of galactose, glucose, and fructose; to a lesser extent, of mannose and dioxyacetone; and practically not at all, of xylose and arabinose. Wilbrandt and Laszt (1933) found a similar arrangement with respect to iodoacetate inhibition, and Laszt (1935) showed that the phosphorylating activity of extracts of the intestinal epithelium exhibited parallel specificity. Verzár (1935) came to

similar conclusions on the basis of the comparative kinetics of absorption of various simple sugars. No extensive series of sugars has been tested with regard to the apparent transport mechanism in the red cell; however, inhibition by mercuric ion, at 1–$5 \cdot 10^{-6}$ M, as described for glucose uptake, is equally effective with the slightly slower levulose, and the slightly more rapid galactose uptake. More complete investigation of the molecular specificity of the relations described in this report is planned, together with tests, suggested by
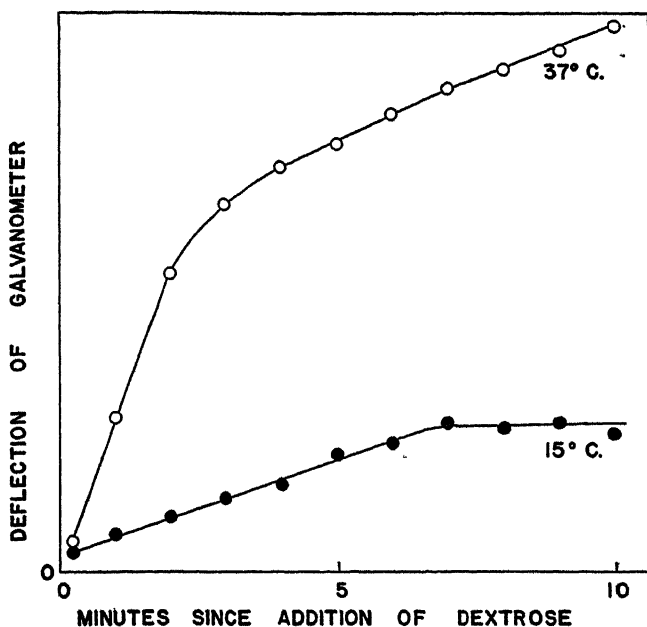
FIG. 10. Effect of temperature on rate of volume changes in glucose-saline solutions. At zero time, 1 ml. 1 M dextrose, in saline medium, added to 5 ml. cell suspension. Deflections plotted as in Fig. 1.

analogy with the intestinal and renal transport, of the possibility of competition between alternative penetrants (Cori, 1926; Shannon, 1938), the influence of hormones (Althausen and Stockholm, 1938), and of various glucosides (Abderhalden and Effkemann, 1934).

The temperature coefficient of the rate at which the cells take up glucose is indicated by a comparison of the initial slopes of the curves in Fig. 10. The Q_{10} computed from these and similar data is about 2.5, which is on the order of that reported by Bjering, although Bang and Ørskov (1937) found a figure of 5.5 with smaller glucose concentrations. Ørskov (1935) also reports a Q_{10} of about 2 for the uptake of glycerol by the human red cell, although Jacobs and coworkers (1935) found a very low Q_{10} for the same process. The figure on the

order of 2–3 would be compatible with the hypothesis of phosphorylation at the membrane as a prerequisite of penetration. However, there is some doubt as to the justifiability of comparing the initial rates of volume change under the same conditions at different temperatures, since the argument developed in the section on kinetics indicates probable involvement of other factors that may vary with temperature. Preliminary experiments indicate that the critical limiting concentration does in fact increase with the temperature, so that, according to the favored hypothesis, the effective gradient for glucose $(L—S/V)$, in the same solution, increases with the temperature (compare Fig. 10). This may account for the extremely high Q_{10} reported by Bang and Ørskov, since comparison of the over-all rates at two temperatures would give a temperature coefficient larger than that of the permeability process itself, as indicated by changes with temperature in the value of "k", the magnitude of the discrepancy depending on the concentration of glucose used in the tests.

SUMMARY

1. Permeability of the human erythrocyte to glycerol, as indicated by the course of hemolysis and volume changes, is depressed by Cu^{++}, Hg^{++}, I_2, p-chloromercuribenzoate, and phlorhizin, without effecting general permeability changes. In so far as tested (Cu^{++}, p-ClHgB), these inhibitors delay exit of glycerol from the cell as well as its entry.

2. Permeability to glucose is similarly depressed by I_2 and phlorhizin, and is extremely sensitive to Hg^{++} and p-chloromercuribenzoate, but is not affected by Cu^{++}. An extensive series of other enzyme poisons is without effect in either system.

3. The effects of the sulfhydryl inhibitors are prevented or reversed in the presence of glutathione, cysteine, etc.

4. The kinetics of the volume changes in glucose-saline solutions indicates a mechanism for transport of glucose into the cell, regulated by the existing intracellular concentration, rather than by simple diffusion gradients.

5. The intermediation of a sulfhydryl group at the cell surface, probably an enzymatic phosphorylation, is suggested as an essential step in the passage of glycerol, glucose, and other like substances, across the human red cell membrane.

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INTRACELLULAR GROWTH OF BACTERIOPHAGE STUDIED BY ROENTGEN IRRADIATION

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The experimental studies carried out on virus particles extracted from infected cells and tissues supply information concerning only the particles that have withstood the extraction treatments. The state of virus particles inside the host cell may be quite different from that of free particles (Pirie, 1946). Moreover, studies of extracts can yield only "static" data, such as facts concerning morphology, chemical constitution, serological properties, etc. They give no approach to virus physiology and disclose nothing about the behavior of the infective virus inside its host. In particular, the key problem of virus multiplication eludes such methods, and this makes it necessary to devise new techniques for the observation of the virus in its natural state.

Electron microscopy has already permitted the observation of virus particles within tissue cultures, whose cells are thin enough to provide a fair transparency to the electrons of 50 to 80 kv. (Claude, 1947). Certain biochemical studies of infected cells also supply information about the behavior of the virus (Cohen, 1947).

A new method, using radiation, was recently suggested by Luria and Latarjet (1947)[1] and applied to the study of bacteriophage T2. The results which were obtained by means of ultraviolet irradiation showed that the method deserved to be extended, and that x-rays might lead to progress along the new path that radiation seemed to offer. In particular, it appeared that one could hope to obtain some answers to the following basic questions: (*a*) What happens to a virus particle once it has been absorbed by a cell? Does it remain as a unit? (*b*) If it remains as a unit, and if multiplication starts from this unit, is it possible to count the number of units present in a given cell at any one time during multiplication, and to obtain thereby a picture of the kinetics of multiplication?

After referring briefly to the theoretical and experimental basis of the method, and the first results reported in Paper I, this paper will describe some new experiments performed with x-rays on the same bacteriophage, and will discuss the results in respect to the two main lines pointed out above.

I

Method

The method, already described in Paper I, is based on the following considerations.

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[1] Hereafter this paper will be referred to as Paper I.

(*a*) One bacteriophage is inactivated by x-rays or by short ultraviolet rays according to an elementary photochemical process initiated by a single quantum of radiation. This is proved by the fact that if one irradiates a homogeneous population of free phage, the survival ratio is a logarithmic function of the dose of radiation. With semilogarithmic coordinates, the survival curve is a straight line (Fig. 2). Inactivation is produced either by a single ionization (x-rays) within some sensitive part of the particle (Wollman and Lacassagne, 1940; Holweck *et al.*, 1940) or by a single short ultraviolet photon (Latarjet and Wahl, 1945).[2]

(*b*) After having received a high dose of radiation, an infected bacterium is still capable of supporting phage growth and of liberating the active particles which it contains. It is therefore possible, by plating an irradiated infected bacterium with an excess of sensitive bacteria, to check whether it still liberates some active virus. (Virus growth within cells sterilized by radiation appears possible also in the case of animal viruses, such as Shope's papilloma virus— Friedewald and Anderson, 1943.)

(*c*) Evidence was given in Paper I that radiation suppresses the infective power of an infected bacterium (let us call the latter an "infective center"), by directly inactivating the virus that it contains. Immediately after single infection (one particle per bacterium), before any multiplication takes place, the survival curve of the infective centers is very similar to that of the extra-cellular virus, and is unrelated to that of the bacterium. In the case of multiple infection (several particles per bacterium) the survival curve of the infective centers is a "multiple-hit" curve with an initial plateau whose multiplicity closely corresponds to the average number of phage particles adsorbed per bacterium. The same feature was observed with x-rays (Fig. 1).

The method therefore consists in infecting a homogeneous bacterial population with a known average number of particles of a given virus, and in irradiating it with increasing doses at a given time during the growth process (latent period); then in determining, for each dose, the survival of the infective centers, and in drawing the survival curve. As the radiation acts directly upon the intracellular virus, such a curve, whose shape is related to the number of virus units actually present in every cell and to their individual sensitivity to radiation, might supply some information about the condition of the intracellular virus at the time of irradiation.

The growth of virus T2 in *Escherichia coli*, strain B, was first studied with ultraviolet rays (Paper I). Survival curves were drawn from minute to minute throughout the 21 minute latent period. Soon after infection, the resistance to radiation of the infective centers increased continuously either because of some structural change of the virus or because of the formation of some ultraviolet-absorbing material in the host which accumulated around the virus and protected it against radiation. Later on, probably after 7 minutes, multiplication seemed to play a rôle in the increase in

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[2] For more details about the radiobiological method, see Lea, D. E., The Action of Radiation on Living Cells, Cambridge University Press, 1945, or Latarjet (1946).

resistance. Still later, after about 12 minutes, the resistance of the infective centers to high doses of radiation decreased, as if, as phage multiplication proceeded, the apparent sensitivity of the virus particles returned to higher values. But a quantitative analysis of the survival curves was made impossible chiefly by the changes in sensitivity of the individual particles during growth. If the latter were actually due to changes in ultraviolet-absorbing material of the host, the use of x-rays would remove this obstacle. As a matter of fact, rapid synthesis of protein and nucleic acid occurs in *E. coli* infected with T2 virus (Cohen, 1947), which might greatly influence
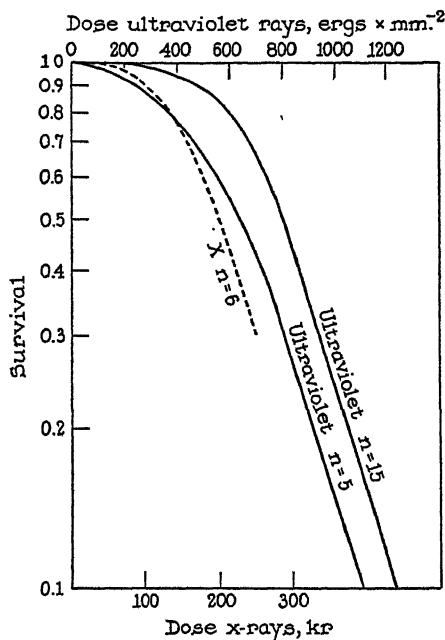
FIG. 1. Survival curves at 5 minutes in the case of multiple infection system, with ultraviolet rays and x-rays.

the ultraviolet experiments, whereas x-ray absorption might remain unchanged. The use of x-rays would therefore eliminate this disturbing factor.

II

Technique of X-Ray Experiments

As in Paper I, *Escherichia coli*, strain B, and bacteriophage T2 were used. Both were grown in a synthetic medium (see Paper I) to which 10 per cent of ordinary nutrient broth was added. In this medium at 37°C., in the presence of a young growing culture of strain B, whose division time is 21 minutes, T2 has a latent period of 21 minutes between the time of infection and the beginning of liberation. Liberation is almost complete 35 minutes after infection; the average phage yield per bacterium is 130. In order to get more time for giving high doses of radiation, some

experiments were carried out at 29°C., a temperature which lengthens the latent period to 43 minutes, while keeping the yield unchanged.

The technique for irradiation of growing phage was the same as that described in Paper I. Powerful specific anti-T2 rabbit serum was used after infection to eliminate free phage (see Appendix).

Because of the high resistance of phage to x-rays, very large doses of radiation had to be given, and, as the main purpose was to draw the survival curve almost from minute to minute during the short latent period, these high doses had to be given in very short exposures. The x-ray set-up was thus devised so as to reach intensities as high as possible with a beam hard enough to penetrate the samples without much loss. The source was a Holweck-Beaudouin tube with a water-cooled molybdenum target. The operating voltage was 33 kv., and the amperage could reach 45 ma. The output, filtered with 0.05 mm. aluminium, included the K_α lines of molybdenum (0.71 A) and some continuous background. The average absorption was that corresponding to 0.95 A, the energy absorbed in a water layer 2 mm. in thickness being about 50 per cent.

Samples of infected bacteria were placed close to the window of the tube in small plastic dishes 17 mm. in diameter and 2 mm. in depth. With an amperage of 43 ma. in the tube, the intensity of the incident beam on the surface of the sample was 90,000 roentgens per minute. A surface dose of 1000 r left in the sample an average of 1.14 × 10^{15} ionizations per ml., a dose equivalent to an incident dose of 715 r on a non-absorbing layer. In the following paragraphs all dosages will be expressed in such effective doses, i.e. in number of ionizations per volume unit in the sample; 1000 r will mean 1.6 × 10^{15} ionizations per ml. In the conditions described above, these 1000 r correspond to 1400 r at the surface, as indicated by a standard ionization chamber.[3]

Protection against Indirect Action of X-Rays.—When one applies high doses of x-rays to biological material suspended in aqueous media, one must deal in general with some indirect action which interferes with the direct action due to the radiant energy absorbed within the biological material itself. This indirect action is due to photochemical effects upon the medium and liberation of toxic substances such as activated O_2, H_2O_2, H^+, OH^-. These effects have been observed in many instances with viruses suspended in aqueous media (Friedewald and Anderson, 1941; Luria and Exner, 1941; Bonét-Maury, 1941; Lea et al., 1944).

In the present work, such indirect action must be carefully avoided, for the method described above is based on the assumption that phage inactivation is due to direct absorption of radiation by the phage itself. Fortunately, virus can be somewhat protected against indirect action by the presence of various substances, which, acting as buffers, fix the by-products of irradiated water.

As a preliminary step, the behavior of T2 was carefully studied. The inactivation of free T2 follows an exponential rate (Fig. 2),[4] but in any aqueous medium indirect

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[3] Calibration was done by Dr. M. Frilley, using the standard large chamber of the Institute of Radium.

[4] $D_{0.37} = 34$ kr. The sensitive volume (target) is equivalent to a sphere 33 mμ in diameter.

action appears when the dose exceeds 100 kr.[5] From that dose on, the survival curve shows a downward concavity. Addition of 10 per cent of ordinary broth counteracts this action until doses of 250 to 300 kr are reached. For this reason broth was added to the synthetic medium in the present experiments. Moreover, the cytoplasm was also expected to protect the intracellular phage. As a matter of fact, no detectable indirect action appeared to disturb the survival curves of infective centers until doses above 400 kr were reached.

<div align="center">III</div>

Experimental Results (Single Infection)

The present work was devoted chiefly to the case of single infection, which appeared more appropriate for providing a fair picture of virus growth. In order to reduce mutual reactivation of inactive particles within the same cell, which may transform single infection into multiple infection (Luria, 1947), only a small fraction of the bacteria were infected with a small amount of phage lysate (low single infection); in general, 7×10^6 phage particles were added to 7×10^7 growing bacteria (per ml.), and the time of contact was reduced to 1.5 minutes. Then, antiserum was added which inactivated almost all non-adsorbed virus. After suitable dilution, a sample was taken at a given time and irradiated. Survival was determined by immediate plating on agar with an excess of strain B, and plaque count. In general, the highest doses given at 37°C. did not exceed 3 minutes of exposure. The question arose whether during such an exposure the infective centers continue to evolve normally or not; that is, whether they are "fixed," by the radiation, in their state at the beginning of the exposure. As will be observed later, the growth system evolves so rapidly at certain times of the latent period that the results of exposures 3 minutes long would be meaningless in the case of non-stabilization of the system. This question was previously considered in the case of ultraviolet rays (Paper I), and it was found that the same dose given either in a few seconds or in an exposure as long as 2 minutes yielded the same results. Moreover, with x-rays, even the dose usually delivered in 1 minute (about 60 kr) disturbs the growth process to such an extent that the latent period is lengthened to 35 minutes, and the individual yield is reduced to a few particles. For these two reasons it was considered that the results given by a 1 to 3 minute exposure were apt to show the situation at the beginning of irradiation. However, when higher doses had to be given, the experiment was carried out at 29°C.

The individual results obtained in successive experiments with a certain dose given at a certain time show considerable variation because of difficulties in maintaining constant all biological and physical factors. Their wide variation makes it necessary to increase the number of individual experiments and to

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[5] The symbol kr will be used for kiloroentgen.

average the results.   The great number of values required to draw the whole
set of survival curves made it advisable to use a graphic average rather than to

TABLE I

Sets of Experimental Data Obtained at Three Different Times during the Latent Period

Time	Dose	Survival
	kr	*per cent*
5 min.		
	20	70
	40	30–46
	56	15–23–20–28
	66	34–20
	114	11–8
	134	17–12
	170	4–5
	200	7–0.2
	230	3–4
	270	2–0.2
10 min.		
	28	94–93–96
	43	85
	57	86–91–90–90–97–91–97
	114	78–67–93–95–92–96
	170	68–56–65
	230	64–39–45–75–41
	340	11–21–18–20–25
	460	4–6–5–5–5
18 min.		
	28	100–102–96–94–95–97
	40	86–84–104–82–95–107–92
	48	98–86
	60	92–80–82–80–90–98–85–98–86
	96	69–78
	115	70–87–79
	130	57
	170	29–47
	230	13
	285	7–5

calculate for each single point a numerical average, whose standard deviation
would be very uncertain.

Preliminary results were recently published (Latarjet, 1947), based on about
200 experimental values.   They showed clearly some new features in the growth
process which could lead to a tentative hypothesis for this process.   But con-
sideration of these results showed that such an hypothesis depended basically

on some details of the curves, and that these details had to be worked out more thoroughly. A new series of experiments was therefore undertaken, particularly with high doses at low temperature. Altogether, 280 data were recorded. As the standard deviation has not been determined accurately for each point, the dispersion of the individual results and the precision of the curves are illustrated by the data in Table I, which gives, as examples, the complete sets of data for three curves. Finally, all results obtained in the case of low single infection were grouped and averaged, and the corresponding survival curves

TABLE II

Average Values of All Experimental Results at Various Times during the Latent Period
(Low Single Infection)

The numbers express in per cent the survival of x-rayed infective centers as a function of the radiation dose.

Dose	Extra-cellular phage	Time of irradiation, *min.*							
		4, 5, 6	8	9	10	11.5	13	15	18
kr									
25	56	56	70	76	98	99	100	99	99
50	30	30	47	60	94	98	100	98	98
75	17	17	32	52	90	95	99	96	92
100	9	10	22	48	85	91	98	92	82
125	5	7.6	17	43	80	88	94	78	68
150	2.8	5.5	13	40	74	83	83	58	50
200	0.8	2.9	7.5	35	58	70	47	29	23
250	0.24	1.5	4.3		42	53	24	14	10
300	0.09				27	36	13	7	4.3
350					15	22	7	3.5	
400					9	14	3.5		
450					5.5	8.5	1.9		

drawn (Table II and Fig. 2). A survey of these results and curves leads us to divide the latent period into three periods:

First Period: First 7 Minutes.—The first characteristic feature is that the resistance of the infective centers to radiation remains constant during the first 6 to 7 minutes, and equal to that of the extracellular virus, at least for low doses. Table III gives as an example the results of an experiment in which a dose of 56 kr was given from minute to minute. For doses higher than 80 kr, *i.e.* for survival below 10 per cent, the remaining infective centers at 5 and 6 minutes appear to be a little more resistant than the extracellular virus, as if the process which, from 7 minutes on, increases the resistance in most of the bacteria had started earlier in some of them.

Second period: from 7th to 13th Minute.—Around 7 minutes (Table III) a slight increase in resistance appears, which becomes more marked as time goes

on. The survival curves at 8 and 9 minutes display this general increase in resistance, with an upward concavity and no plateau; *i.e.*, no feature of multiplicity (compare with Fig. 1). On the other hand, at 10 minutes an entirely
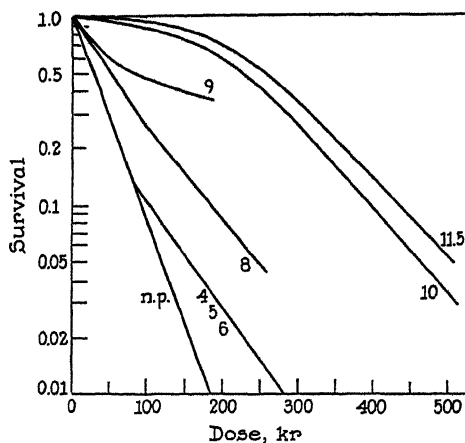
FIG. 2 a
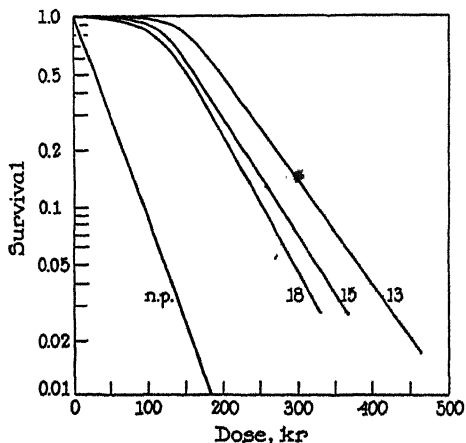
FIG. 2 b

FIG. 2. Survival curves of low single infective centers. (*a*) From 4 minutes to 11.5 minutes; (*b*) from 13 minutes to 18 minutes. Curve *n.p.* is that of extracellular T2.

new picture appears, with a greatly increased resistance, a downward concavity, and some kind of an initial plateau. At 11.5 minutes these new features are even more marked, at which time the resistance of the infective centers to high doses of radiation has reached its maximum.

Third Period: from 13 Minutes to Burst.—At 13 minutes the survival curve is definitely of a multiple-hit type. It shows a long plateau, a downward concavity, and finally, a straight portion. Such a curve can be analyzed for multiplicity (see Discussion). From this time on, the survival curve keeps its general shape, but the sensitivity to radiation increases slowly until the end of the latent period. At 18 minutes, the straight part of the curve is parallel to the curve of extracellular phage.

It should be pointed out that the general picture obtained with x-rays resembles closely that obtained with ultraviolet (Fig. 3). The differences will be emphasized in the Discussion.

IV

Comparison of Low and High Single Infections

It was found with ultraviolet rays (Paper I) that the amount of phage lysate introduced into the bacterial suspension influences the resistance of the infective

TABLE III

Survival during the First Period, after a Dose of 56 Kr

Time of irradiation	Survival
min.	*per cent*
(Extracellular virus)	24.5
4	24
5	23
6	24.5
7	33
8	55

centers to radiation during the first 10 to 12 minutes, even in the case of single infections. The greater the amount, the faster the resistance increases. New experiments with x-rays confirmed what had been found with ultraviolet rays.

The usual bacterial suspension containing about 7×10^7 bacteria per ml. was infected with 5×10^7 T2 per ml. The number of infective centers was about 3.5×10^7, indicating that about 1 bacterium out of 2 (high single infection) was infected, whereas in the case of low single infection 1 out of 10 to 20 bacteria was infected. In both cases, a majority of the infected bacteria were "single infected," and all other experimental conditions were the same, the only difference being the amount of phage lysate introduced.

Fig. 4 shows the survival curves obtained in the case of high single infection during the first 10 minutes of the latent period, and compares them to some of the low single curves of Fig. 2. Here again the process which increases the resistance of the infective centers starts earlier in the case of high single infection, the difference between the two cases being 2 to 3 minutes. For instance,
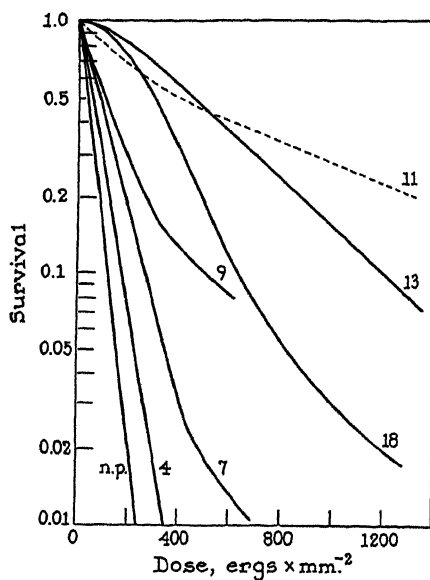
FIG. 3. Survival curves of low single infective centers in the case of ultraviolet irradiation (from Paper I).
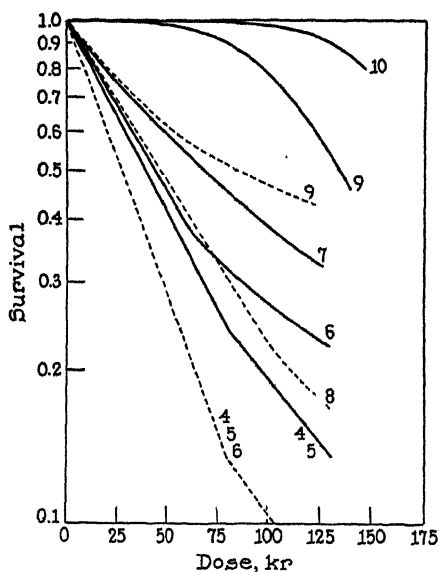
FIG. 4. Survival curves during the first 10 minutes, in the case of high single (plain curves) and low single (dotted curves) infections.

at 9 minutes the high single curve is already of the multiple-hit type, and the 10 minute curve is similar to the 13 minute low single curve. It seems that, in the case of high single infection, the first period is very much reduced, and the second one ends around 10 minutes. From that time on, there is no more difference between low and high single curves.

DISCUSSION

The main purpose of the present work, as already pointed out in Paper I and in the introduction of the present paper, is to clarify the fundamental problem of virus growth, whose importance may be related not only to viruses but also to specific cellular constituents such as microsomes, plastids, genes, and enzymes. It was believed that the knowledge of the kinetics of growth, *i.e.* the variation with time of the number of virus particles in a given cell, would provide the first clue to the problem, and radiation was used chiefly as a possible tool for counting the average number of virus particles within the infected cells of a homogeneous population at any one time between infection and lysis.

If one assumes that the growth process yields only replicas of the initial virus particle, and that it is the same in all cells of the population, the survival curves after irradiation can be theoretically drawn as a function of the number of particles actually present in each cell (Fig. 5; for explanation, see Paper I). A comparison of these theoretical curves with the experimental ones should permit estimations of the number of particles per cell at any one time. On the other hand, if the experimental curves differ significantly from the theoretical ones, one or both preceding assumptions would be ruled out and some new feature could be disclosed in the growth process.

It should be noted that phage multiplication is likely to proceed at varying rates, leading to a wide distribution of the phage yields from individual infected bacteria (Delbrück, 1945). Not all bursts occur at the same time; some cells yield few particles whereas others yield several hundreds. This wide distribution might somewhat distort the curves from their theoretical shape.

Before discussing the preceding results with x-rays, one should keep in mind the chief significance of the theoretical curves drawn in semi-logarithmic coordinates. A straight line means a one-hit killing process.[6] As killing of an infective center results from the inactivation of the infective virus (see section I *c*), a straight line means the presence of only one virus particle per cell. The presence of several identical particles per cell leads to multiple-hit curves (Fig. 1). Such curves (Fig. 5) display an initial plateau whose length is related to the number of particles, and a straight part which is parallel to the one-hit curve. The common slope of all straight parts defines the radiosensitivity of the individual particle: the greater the slope, the higher the sensitivity. In

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[6] In the present case of infected bacteria, the term "killing" is used for convenience, and means loss of ability to liberate active phage.

general, x-ray sensitivity of virus particles is related to their size, at least to the size of a special sensitive volume of their body: the greater this volume, the higher the sensitivity.   Therefore, if the size of the particle increases, the slope of the survival curve may be expected to increase.

We shall now try to interpret the curves obtained in the case of low single infection (Fig. 2 *a* and *b*) during each of the three periods into which these curves lead us to divide the latent period.
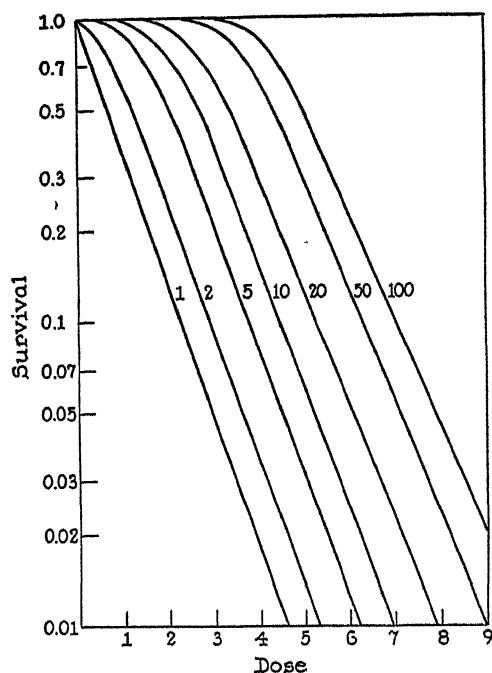
FIG. 5. Theoretical survival curves for different values of the multiplicity, from 1 to 100.

First Period: Bacterial Synthesis.—During the first 7 minutes, the survival curve of the infective centers remains almost identical with that of the extra-cellular virus.   This means that: (*a*) the infective virus particle remains within the cell in such a state that its x-ray sensitivity is unchanged; it remains genetically intact, whether broken into subunits or not; (*b*) this virus remains unique; no multiplication takes place during this period.

The ultraviolet curves (Fig. 3) show that, during this time, the resistance of the infective centers to ultraviolet radiation progressively increases.   It was suggested that this increase was due to bacterial synthesis of a large amount of ultraviolet-absorbing material (Paper I).   The x-ray curves confirm this hy-

pothesis by ruling out a possible change in the radiosensitivity of the virus particle itself.

The chief phenomenon that occurs during the first 7 minutes seems to be the bacterial synthesis of ultraviolet-absorbing material probably devoted to the building of future particles. McFarlane (1947) has shown that epidermal cells infected with vaccinia virus are very rich in proteins with the same antigenic constitution as the proteins of the virus. Cohen and Anderson (1946) and Hook *et al.* (1946) found that virus T2 contains about 35 per cent of desoxyribose nucleic acid. From Cohen's experiments (1947), the synthesis of this acid in the infected cells starts after 7 minutes only. The previous syntheses might then be concerned mainly with protein material.

The first alterations following infection affect the host and not the virus, a fact which may explain the interference phenomenon by changes in the host functions. Working on ultraviolet-irradiated influenza virus, Henle and Henle (1947) reached the same conclusion that the first step after adsorption of the virus onto the host cell would "lead to changes in the host cell which alter its function and exclude other viruses from entering."

Second Period: Multiplication.—Around 7 minutes, the first critical change occurs: a process starts which increases the x-ray resistance of the infective centers, without changing essentially the shape of the survival curves, which remains without any plateau for about 2 minutes (8th and 9th minutes), proving that the multiplicity is still equal to 1. The infective particle, still unique, undergoes some changes which affect its resistance to radiation. The responsible process appears highly heterogeneous within the population (hence the upward concavity of curves 8 and 9, and the break of curves 4 to 6 indicating that in a number of cells the process starts before 7 minutes). Such heterogeneity may be connected with that in individual yields after lysis.

This unknown process is favored by a constituent of the phage lysate different from the active particles themselves, for it goes faster in the case of high single infection, and even faster in multiple infection.

It has been shown with ultraviolet rays, and confirmed with x-rays, that, in the latter case, several virus particles (one does not know yet how many) are capable of growing in the same bacterial cell, a fact which is in accord with Hershey's observation (1946) that bacteria simultaneously infected with T2 wild type and its *r* mutant yield both types. Both latent period and yield are the same as in the case of single infection, although the first steps of the growth process are accelerated, the end of the second period occurring around 11 instead of 13 minutes.

Between 9 and 10 minutes a new phenomenon transforms the survival curve into a multiple-hit type one, with a definite plateau and a straight part whose slope is far weaker than that of the extracellular virus curve. This seems to indicate that: (*a*) some units are multiplying very fast and are already numer-

ous at 10 minutes; (b) these units are more resistant to radiation (probably smaller) than the extracellular particle. The multiplicity increases rapidly and reaches its maximum around 13 minutes, with 100 to 150 virulent units whose x-ray sensitivity is about half that of the initial particle.

Thus multiplication itself seems to proceed for about 4 minutes (9th to 13th minute), and the multiplying units appear less sensitive to x-rays (probably smaller) than the extracellular virus particle.

Third Period: Maturation of Virus Particles.—At 13 minutes, a second critical change occurs: multiplication stops. The number of units has reached its maximum and will not change any further. From this time on, general sensitivity of the infective centers increases until, at the very end, a situation is reached in which an average of 100 to 150 particles are present per cell, displaying the same radiosensitivity as that of the oroginal particle in its free state. This last phase of the growth process is probably devoted to the maturation of the new particles.

These results confirm those obtained with ultraviolet rays, and add some new details to the tentative and crude picture of the sequence of events in T2 growth which was given in Paper I and can now be drawn as follows: After one virus particle has penetrated into a bacterial cell, some ultraviolet-absorbing material, probably needed for virus building, accumulates. Additional stimulus for this phenomenon can be supplied by some other component present in phage lysates besides the active phage itself. For about 7 minutes the infective particle remains unique and genetically intact, its resistance to x-rays being constant. Between 7 and 9 minutes, the particle undergoes some changes which increase this resistance. The whole process so far varies widely in speed or magnitude from cell to cell, and this variation may be partly responsible for the variability of virus multiplication in different cells, reflected in the variability of virus yield. At about 9 minutes, multiplication starts and continues until, about the 13th minute, an average of 100 to 150 infective units are present per cell. The multiplying units are more resistant to x-radiation than the liberated virus; they probably are smaller. In the last part of the latent period, the new infective units undergo some changes (probably grow in size) until, at the very end, they appear similar to the extracellular virus.

As far as the nature of the multiplying units and the rate of multiplication are concerned, nothing as yet can be said. It was impossible to draw, between 9 and 13 minutes, as many survival curves as were needed to get a picture of the kinetics of growth. The present work fails to tell whether the rate is linear, logarithmic, or otherwise; whether multiplication proceeds by some "template mechanism" (linear rate) or by self-duplication of all units behaving like higher microorganisms (logarithmic rate). It fails therefore to answer the fundamental question mentioned in the introduction. But it shows that the multiplying

units are somewhat different from the infective particle, and strongly suggests that they are smaller. It does not rule out Luria's idea, based on his discovery of cross-reactivation between ultraviolet-inactivated T2 particles within the same host cell (1947), according to which the infective particle would break into genetic loci which might reproduce with a certain degree of independence, and then be utilized in the building of new particles.

The present crude picture of virus T2 growth reveals the whole process as highly dependent upon bacterial metabolism. Multiplication starts only after some specific material has been synthesized by the cell, and possibly stops upon exhaustion of some substrate, which plays the rôle of a limiting factor. Bacterial synthesis may continue during the period of multiplication, but it also may be curbed when a certain number of new units are formed. Early deviation of normal bacterial synthesis indicates that, in the competition among cellular elements for enzymes and energy, the infective particle appears as a winner. For instance, it stops normal synthesis of ribose nucleic acid (Cohen, 1947) and the capability of the cell for enzymatic adaptation (Monod and Wollman, 1947). One can imagine that at a certain stage of the growth process, the cell is disturbed to such an extent that it is no longer capable of building the material needed by the multiplying units.

At the time of infection, when viruses come into contact with growing bacteria, the cells are in widely varying stages of their growth; i.e., of their synthetic potentiality. Since the latter seems to be involved in virus growth, this could account, at least partly, for the wide distribution of the individual yields.

SUMMARY

Growing *Escherichia coli* infected with bacteriophage T2 was x-rayed during the 21 minute latent period which elapses between infection and lysis of the cells. Survival curves of the infected bacteria were determined almost from minute to minute; they disclosed the following facts which are related to the process of phage growth:

During the first 7 minutes, the infective virus particle remains in the cell unique and genetically intact. The host cell synthesizes some ultraviolet-absorbing material probably devoted to building future particles. From the 7th to 9th minute the x-ray resistance of the virus particle increases, probably because of some internal change. Then, multiplication starts and is completed at about the 13th minute, when an average of 130 virulent units is present per cell, displaying an x-ray resistance twice as high as that of the extracellular virus particle. From 13 minutes to the end, the new units progressively recover the x-ray sensitivity of the extracellular virus.

Nothing can be said about either the rate of multiplication between 9 and 13 minutes, or the nature of the multiplying units, except that they are more radiation-resistant (probably smaller) than the extracellular virus.

The first steps of the growth process are favored by an unknown component of the lysate, different from the active particles.

Several particles can grow in the same host cell.

I am happy to acknowledge my indebtedness to my assistants P. Morenne and Y. Déziré for their help in carrying out the experiments; to the National Institute of Hygiene, France, for its financial support; and to Dr. S. S. Cohen, Dr. S. E. Luria, and Dr. J. Monod for their advice and criticism.

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APPENDIX

A New Method for Obtaining Powerful Antiphage Rabbit Sera

In the experiments with ultraviolet rays (Paper I) we used an anti-T2 rabbit serum prepared according to Delbrück's technique (1945), which consists of two courses of subcutaneous injections of a high titer lysate. Each course consisted of five injections of 5 ml., given twice a week, and there was an interval of about 1 month between courses. With T2 virus the best serum we obtained in this manner inactivated 99 per cent of free particles in 30 seconds, when diluted tenfold. With smaller phage particles, such as T7, the results were far less satisfactory, probably because the injections provide a much smaller amount of antigenic proteins.

A striking improvement was achieved with both T2 and T7 by (*a*) using intravenous injections in the marginal vein of the rabbit's ear; and (*b*) using a new schedule of injections which was suggested to me by Dr. J. Loiseleur.

High titer filtered lysates in synthetic medium were used (1 to 5 × 10^{10} particles per ml.). A first course was given of ten subcutaneous injections of 2 ml. twice a week. After an interval of 1 month, a second course of intravenous injections was given with the same lysate:

Day	Amount of lysate injected
	ml.
1	0.5
4	0.5
6	0.5
8	1
11	1
13	2
15	2
21	Bleeding

Bleeding was done aseptically by direct intracardiac punction, 20 cc. of blood being withdrawn. This punction was withstood well by the rabbit and could be repeated later on.

Activity.—The T2 antiserum, diluted 50-fold, reduced the titer of a lysate from 1 $\times$ 10^{10} particles per ml. to 5 $\times$ 10^4 in 30 seconds (inactivation of 2 $\times$ 10^5), and achieved complete inactivation of this lysate in 1 minute.

The T7 antiserum gave the same rate of inactivation when diluted tenfold. If compared with the preceding sera, the present ones are from 10^3 to 10^4 times more active.

Storage.—In order to avoid contamination, a small crystal of thymol was added to the serum. Almost insoluble, it remains indefinitely, and the small amount dissolved prevents practically all contamination without affecting the activity. Such a saturated solution of thymol, if diluted fivefold, does not kill T2, T7, or B bacteria in 5 minutes. Under the usual conditions, complete inactivation of the free particles is obtained before any danger of the toxic action of thymol on the phage or on the bacteria.

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Announcement

Volumes 131 and 132 of the *Studies from The Rockefeller Institute for Medical Research* are devoted wholly to the publication of a work by Dr. Rafael Lorente de Nó entitled

A STUDY OF NERVE PHYSIOLOGY

The subject matter consists of the report of experiments for which the *Studies* provides the place of original publication. The volumes appeared in September, 1947, and together contain about 1060 pages with 480 illustrations.

In order that the volumes may be available to those who do not receive them as part of their subscriptions to the *Studies*, extra copies have been printed which are purchasable at the regular price of $2.00 per volume. The two volumes will only be sold together. They can be obtained from the Publication Service, The Rockefeller Institute for Medical Research, York Avenue and 66th Street, New York 21, N. Y., at a price of $4.00, payable in advance.

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